Conference and Workshop on Cellular Immune Reactions to Human Tumor-Associated Antigens

Monograph 37

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Conference and Workshop on Cellular Immune Reactions to Human Tumor-Associated Antigens

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CONFERENCE AND WORKSHOP ON CELLULAR IMMUNE REACTIONS TO HUMAN TUMOR-ASSOCIATED ANTIGENS

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PREFACE

It is now generally accepted that human tumors contain tumor-associated antigens and that one can measure cell-mediated responses to these antigens. In the last 5 years, a variety of assays have been developed to study this type of reactivity. Because of the great interest in these assays and because of the various problems that investigators are having with these assays, the Immunology-Epidemiology Segment of the Special Virus Cancer Program of the National Cancer Institute decided to have a conference to review the state of the art and to discuss the details of the tests and their biologic significance. Some of the questions to be raised at the Conference include: 1) What types of antigens are being detected in the various assays? In any particular assay, are the detected antigens tumor specific or tumor associated or are they tissue antigens or normal antigens expressed in some modified form or perhaps in quantitatively increased amounts on the tumor relative to the normal? 2) What are the phases of the immune response that these assays measure? It is often stated that one is doing either an assay of cellular immunity or of humoral immunity. It is becoming increasingly clear that one cannot just categorize assays like this into neat packages, and that a particular cell-mediated assay may be measuring a part of the immune response quite different from that of another assay of cellular immunity. Each assay may or may not be relevant to the in vivo state of immunity in the patient. 3) What is the nature of the reactive lymphocytes? Are they T-lymphocytes or B-lymphocytes? What role do lymphocyte-dependent antibodies play in the observed responses? 4) What are the relationships of these various assays with each other? If one sets up in parallel 2 or 3 of these assays, would the same answers be obtained with the different assays? 5) How reliable and reproducible are the results of these assays? If one did the same assay one day and then again on the next day, would one get the same results? 6) How useful are these assays? Can any of these assays be applied to immunodiagnosis? Can they be used to differentiate patients with neoplastic diseases from those with benign disease or from normal individuals? Can any of these assays be used for epidemiologic studies, to look at family members of cancer patients or high-risk groups? 7) Lastly, can such assays be used as a monitor of a patient's condition or as a guide to conventional therapy or immunotherapy?

The Conference was organized according to assays. Investigators experienced with a particular assay discussed their techniques and some of their results. This was followed by a detailed discussion, centered around some of the above questions. This discussion was the heart of the meeting, with open discussion on the details of, and problems with, the assays.

Ronald B. Herberman, M.D.
Chairman of the Conference

INTRODUCTION

Recent developments in the field of cell-mediated immunity have indicated that it is possible to detect tumor-associated antigens by a variety of techniques in vivo and in vitro. The importance of these tests in monitoring the patient's response to his own tumor and the possibility of applying these tests to the monitoring of immunotherapy are obvious, but the potential importance of these techniques to studies on the etiology of cancer should not be overlooked. The topic of cell-mediated immunity in human cancer was selected as the first of a series of workshops by the Immunology-Epidemiology Segment of the Special Virus Cancer Program of the National Cancer Institute because of our hope that the immunologic expertise that is being applied to studies critical to the control of cancer can also be applied to studies relevant to etiology. An early example of the potential application of such tests to viral oncology was demonstrated by the Hellströms (*1*) when they showed that parents and siblings of neuroblastoma patients had lymphocytes cytotoxic for neuroblastoma cells, while families of patients with Wilms' and other pediatric neoplasms had lymphocytes not cytotoxic for neuroblastoma cells.

We had the opportunity to pursue similar studies in patients with acute leukemia with an additional handle on the specificity of the reaction when we evaluated leukemic patients who had clinically identical twins (*2*). Our initial impetus was provided by early reports that antisera prepared against murine leukemia viruses could detect antigens in the cytoplasm of human leukemic cells (*3, 4*), but we intended to exploit the opportunity to study essentially syngeneic controls by using as many different types of tests as possible. To date we have studied 10 such twins in a collaborative project involving more than 8 investigators and 4 tests of cell-mediated immunity. Eight of our patients had acute lymphocytic leukemia and two had acute myelogenous leukemia; only one set of twins was >15 years old at the time of study.

One test, the lymphocyte-mediated cytotoxicity assay (*5, 6*), which Dr. Herberman and Dr. McCoy will discuss later in this Conference, was particularly interesting, since cytotoxic lymphocytes from family members and related individuals were found to attack the cells of the leukemic patients but not those of the normal twins in 11 of the 20 experiments (table 1). Skin tests, mixed lymphocyte culture, and macrophage migration-inhibition (*2, 7*) also appeared to detect leukemia-associated antigens in the patients' cells which were not found on the cells of the normal identical twins. But only the lymphocyte cytotoxicity tests remained positive when the patients were in clinical remission. The finding of reactivity in the family members and in other individuals of the general population against the leukemic twin's cells but not against the normal cells indicates that normal individuals have been sensitized against some antigen associated with acute leukemia, and a virus is one possible sensitizing agent that must be considered.

TABLE 1.—*Effect of cytotoxic lymphocytes from family members and unrelated individuals on leukemic and normal cells*

Patient	Date of diagnosis	Date of study	Status at time of study	Target cells	Attacker cells				
					Twin	Mother	Father	Sibling	Unrelated individual
H	12/68	12/29/69	Remission	Patient	0.9	11.9*	11.9*	16.2, 3.9	
				Twin	0.8	0.3	−0.9	0.9, 3.4	
		12/ 8/70	Remission	Patient	−0.3	4.3*	4.2*	2.7,* 3.9	2.3,* 2.8,* 3.0*
				Twin	−0.9	0.9	−0.8	−0.2, 2.4	0.4, −0.3, 1.4
B	8/69	2/ 2/70	Remission	Patient	−0.8	−1.2	−1.6	0.9, 0.2	
				Twin	0.9	−1.5	−0.1	−0.4, 0	
		3/16/71	Remission	Patient	−0.6		0.2	−1.1, 0.1	0.7*
				Twin	0.3		0.5	−1.0, 0.6	1.6*
D	6/67	5/12/70	Remission	Patient	−1.8	−1.9	−1.4	−2.9	−0.8, −0.3
				Twin	−3.7	−5.6	−4.2	−0.9	−3.6, −2.5
L	9/69	10/23/70	Relapse	Patient	1.6	8.4*	1.7		3.2
				Twin		4.7	5.0		−1.2
		8/19/71	Remission	Patient	0.9		3.2	13.5	0.9, 3.7
				Twin	−5.3		−3.9	−2.3	−3.9, −6.4
M	10/70	10/27/70	Untreated	Patient	2.4*	2.5	0.6	1.0	2.9*
				Twin	−5.1	−4.4	−2.8	−2.6	−0.5
		2/ 9/71	Remission	Patient	−1.3	5.8*	−1.1	−0.4	4.9*
				Twin	−0.7	11.1*	−0.5	1.3	0.5
V	5/70	11/18/70	Remission	Patient	0	1.1*	0.8*	Not done	1.1,* −0.3, 0.2
				Twin	0.2	2.3*	3.9*	Not done	3.0,* −0.7, 0.3
		7/18/71	Remission	Patient	0	0.4	0	Not done	−0.3, 2.3,* 0.2, 2.4,* −1.3
				Twin	−0.1	0.2	0.1	Not done	0.7, 3.3,* 0.8, 8.6,* 0.5
A	7/70	2/17/71	Remission	Patient	0.2	0.8*		1.2*	2.3*
				Twin	0.8	2.5		−0.9	0.9
		6/ 1/71	Remission	Patient	−1.5				−1.7, 0.3, −8
				Twin	−0.3				−1.2, 0.6, −1.2
C	1/71	5/ 4/71	Remission	Patient	0.9*	0.6	0.7*	0.6	1.1,* 0, −0.5
				Twin	0.5	1.0	−0.5	0	0.1, 0.2, 0.2
G	5/71	6/25/71	Remission	Patient					0.1, 1.0, −0.9, 0.8, 27.7* 2.8
				Twin					2.5, 2.2, −0.1, 1.3, 14.0* −1.4
		7/29/71	Partial remission	Patient	0.9	3.9*	1.4*	−1.7,* 1.5,* 1.7, 1.3, 1.5	0.7, 0.1
				Twin	1.9	2.2	2.9	0.2, 2.1, 0.2, 1.1, 2.4	−1.3, 2.5
		10/28/71	Remission	Patient	9.3*	1.1	4.4*	5.9,* 0.9,	−1.7, 0.5

				Twin	−0.7	−4.1	1.3	−0.2, 5.1 1.0, −2.3, −1.2, −2.0	−0.4, −1.4
		2/ 7/72	Remission	Patient	−1.9	−1.5	−0.2	0.1	−4.8, −0.3
				Twin		4.4	5.6	−3.5	6.7
N	9/71	9/10/71	Untreated	Patient†	13.9*	9.1			3.3*
				Twin†	2.0*	0.6			0.3
		2/ 7/72	Remission	Patient	1.4	1.1			4.3, 1.0. −2.9, −0.9
				Twin	2.9	2.2			−0.9, 2.7

*Percent lysis of cells in this reaction is significantly above control values ($P<0.05$).
†Target cells were obtained from bone marrow in this experiment.

Our current studies are now being directed to the specificity of these reactions, and there is some evidence that more than one leukemia-associated antigen is being detected. Indeed, it was the difficulty in reconciling some of the differences among the immunologic tests used by Dr. Herberman, Dr. Halterman, and others involved in this study and similar studies that helped to stimulate the development of this workshop.

The problem of identifying which antigens these tests are detecting is an important one. Progress in the separation of tumor-associated antigens may be expected to provide us with important information similar to the data that have been unfolding in the studies of Burkitt's lymphoma (BL) over the past 8 years *(8–10)*. Separation of some of the EBV-associated antigens has permitted an opportunity to identify some which produce antibodies relevant to prognosis *(11, 12)* and even now it is quite likely that further separation of some of these antigens must be done *(13)*. Although two of these antibodies, those directed against the EBV-associated early antigens and membrane antigens, may prove to be useful in predicting survival, the role of the virus in the etiology of BL has not been clarified. Hopefully, through this workshop and similar workshops, investigators in viral oncology and tumor immunology will be able to pool their efforts to develop tests which, through their detection of specific antigens, can be applied to studies on the etiology as well as the diagnosis and control of cancer.

Paul H. Levine, M.D.
Chairman
Immunology-Epidemiology Segment

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PART I

CYTOTOXICITY ASSAYS

Tumor Immunity in Melanoma Patients as Assessed by Colony Inhibition and Microcytotoxicity Methods: A Preliminary Report [1, 2]

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SUMMARY—Sequential data are presented on cell-mediated immunity (CMI) and serum-blocking factor (SBF) against melanoma cells in melanoma patients. The methods of assay were a dilute agar colony-inhibition test and a microcytotoxicity test. CMI, found fairly constantly in patients with stable disease, may be undetectable in those whose disease is widespread or who have undergone surgery within a week of the test. Although detection of SBF is generally associated with a poor prognosis and failure of detection is a good sign, SBF may be found in patients without, and be absent in those with, active disease. Frequent assessment of SBF is thus necessary. The significance of these data to the individual cancer patient is discussed.—Natl Cancer Inst Monogr 37: 3–8, 1973.

COLONY INHIBITION (CI) and newer microcytotoxicity (MC) assays detect cell-mediated immunity (CMI) and associated humoral antitumor immunity in patients with a variety of tumors (*1–5*). The immune responses detected by such in vitro methods seem related to in vivo events, as shown by experiments with animals and human cancer patients. CMI is a fairly constant finding, although in some systems it wanes with increasing tumor load (*6*). Serum-blocking factors (SBF), presumably antibody or antigen-antibody complexes (*7*) capable of blocking cell-mediated immunity, are more restricted in occurrence and are found most often in patients with active, spreading disease and less frequently in those with local or clinically inactive disease (*2, 8*). Thus the monitoring of CMI and SBF in individual cancer patients might help the physician to assess the efficacy of treatment and the prognosis of the patient. However, there are many technical difficulties in detecting CMI and SBF, and it may not be possible to do so regularly enough to aid treatment of the individual patient.

We present here preliminary, sequential data on detection of CMI and SBF in patients with malignant melanoma. These data are selected from a larger study which will be presented elsewhere. However, we think that they are typical of the larger series and will illustrate our results.

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[8] We thank Mrs. Janice Kopp and Miss Elizabeth Swanson for their expert technical assistance.

MATERIALS AND METHODS

Cell culture and test materials.—All cell culture reagents were obtained from Grand Island Biological Co., Grand Island, New York. Plastic culture flasks, tubes, petri dishes, and Microtest II plates came from Falcon Plastics, Oxnard, California. Plasmagel was purchased from Laboratoire Roger Bellon, Neuilly, France.

Cultivation of tumor cells.—Tumor cells were cultured from biopsy samples. For the dilute agar test, the cells were grown in agar suspension culture, as described by Heppner and Kopp (*9*); Waymouth's medium supplemented with 20% horse serum was used. For the MC assay, the cells were grown in monolayer culture; Waymouth's medium supplemented with 20% fetal calf serum was used. For some tests we used the BERO cell line, kindly supplied by Drs. David Clark and Larry Nathanson, Department of Medicine, New England Medical Center Hospitals, Tufts University School of Medicine, Boston, Massachusetts. This cell line, grown in monolayer culture, is an aneuploid, dopamine-positive line from a blood group O patient.

Classification of melanoma patients.—The patients were classified according to extent of disease, as follows: 1) local—disease involving primary site without evidence of metastasis to lymph nodes; 2) regional—disease involving primary site plus local nodal drainage area, or recurrent disease within that area; or 3) widespread—disease which has spread beyond nodal drainage area, either subcutaneous or visceral.

Lymphocytes.—Lymphocytes were obtained from peripheral blood collected in sterile vacutainer tubes containing 286 USP units of sodium heparin. Granulocytes were removed by settling on glass, and red blood cells by sedimentation through plasmagel, as described by Hellström et al. (*1*).

Lymphocytes were collected from melanoma patients and control donors. The latter were healthy adults, approximately 20–50 years old, and were mainly secretaries, laboratory and maintenance personnel, and physicians.

Dilute agar CI test (GEL).—The method for this CI test has been described previously (*9*). Lymphocytes were added to target tumor cells in a ratio of 100:1. Serum for blocking activity was assayed at a dilution of 1:2.

MC assay.—The method used was that described by Hellström et al. (*1*) for Falcon #3040 Microtest II plates. Lymphocytes were added to target tumor cells in a ratio of 1,000–5,000:1. Serum for blocking activity was assayed at a dilution of 1:6.

Statistical analysis.—All data were analyzed by the Student's *t* test (*10*).

RESULTS

Detection of CMI and SBF in Patients With Stable Disease

For monitoring of CMI and SBF in a tumor patient to be of value to the physician, changes in these responses will have to correlate with changes in clinical status. Conversely, stability in immune responsiveness should signify stability of disease. Tables 1 and 2 present data on detection of CMI and SBF in patients whose disease picture did not change during the testing period. Repeated tests were done at times ranging from the same day to 9 months after the initial observation. The tests on the same day were performed with different tumor target cells, or in the case of SBF, different immune lymphocytes. Both GEL and MC tests were done.

Detection of CMI (table 1) remained constant during the testing periods. For the most part, the level of CMI remained constant also, although it dropped in patient P.B. Detection of SBF was more variable (table 2). Thus patient I.B. was negative for SBF on 10/4/71 but positive on 2/2/72, even though her disease had seemingly not reappeared by May 1972—5 years after surgical removal of a localized lesion on the left leg.

Detection of CMI and SBF in Patients With Changing Disease

Included in the category of patients with changing disease are only those in whom major shifts in clinical status have occurred. In table 3 are sequential data on patients who have steadily deteriorated from widespread disease to death during the testing period (patients W.K.,

TABLE 1.—*CMI to melanoma cells in patients with stable disease*

Extent of disease	Patient	Target cell donor	Date of test	Type of test	Average No. of colonies or cells		Percent significant CMI
					Control's lymphocytes	Patient's lymphocytes	
Local	A.T.	A.T.	7/21/71	GEL	23.1±4.1	13.5±2.1	41.6*
		M.T.	4/25/72	MC	24.5±5.4	15.8±6.7	35.6†
	M.M.	W.K.	4/16/71	GEL	26.0±0.8	16.5±3.1	35.0*
		B.B.	4/16/71	GEL	24.3±3.3	17.0±1.4	30.1*
	P.B.	M.T.	2/2/72	MC	30.8±6.4	5.9±2.4	80.8*
		M.T.	2/7/72	MC	23.8±3.7	2.1±0.9	92.2*
		M.T.	3/8/72	MC	57.3±6.9	25.3±6.8	56.1*
Regional	H.T.	M.T.	12/1/71	MC	17.0±8.4	9.2±1.0	45.9*
		M.T.	3/27/72	MC	6.4±2.1	1.0±0.0	84.4*
Widespread	H.G.	B.B.	4/16/71	GEL	20.0±2.4	13.5±3.1	32.5*
		G.U.	5/19/71	GEL	10.3±1.5	6.8±2.4	34.0†
		A.J.	5/19/71	GEL	12.3±1.5	8.3±0.5	32.6*

*$P<0.005$.
†$P<0.05$.

TABLE 2.—*Blocking immunity to melanoma cells in patients with stable disease*

Extent of disease	Patient	Immune lympho-cyte donor	Target cell donor	Date of test	Type of test	Average No. of colonies or cells with immune lymphocytes and		Percent significant SBF
						Control's serum	Patient's serum	
Local	J.A.	J.A	M.T.	4/10/72	MC	3.0±2.7	2.0±0.8	0.0
		R.C.	M.T.	4/10/72	MC	7.3±5.1	3.8±2.5	0.0
	A.T.	A.T.	A.T.	7/21/71	GEL	29.3±0.6	10.7±2.5	0.0*
		J.B.	A.J.	7/21/71	GEL	8.3±2.6	10.0±0.0	0.0
		A.T.	M.T.	4/25/72	MC	15.8±6.7	23.8±8.1	0.0
	I.B.	J.B.	BERO	10/4/71	MC	5.5±1.9	5.5±1.3	0.0
		I.B.	M.T.	2/2/72	MC	9.5±3.4	16.0±0.0	68.4*
Widespread	J.B.	J.B.	A.J.	8/2/71	GEL	10.7±3.1	21.0±0.0	96.2*
		J.B.	M.T.	4/4/72	MC	28.8±4.5	38.5±1.9	33.3*

*$P<0.05$.

TABLE 3.—*CMI and blocking immunity in patients with rapidly advancing melanoma*

Patient	Date of test	Type of test	Target cell donor	Percent significant CMI	Immune lymphocyte donor (SBF test)	Percent significant SBF
W.K.	8/11/70	GEL	W.K.	38.3*	W.K.	40.8†
	3/19/71‡	GEL	W.K.	0.0	E.N.	0.0
A.J.	5/19/71	GEL	A.J.	42.4*	A.J.	31.6*
	7/21/71‡	GEL	A.T.	0.0	—	—
H.G.	4/16/71	GEL	B.B.	32.5*	H.G.	66.7*
	5/19/71	GEL	A.J.	32.6*	H.G.	57.9*
	7/28/71‡	GEL	A.T.	41.6*	H.G.	0.0
H.R.	1/27/72	MC	M.T.	43.5*	—	—
	2/16/72‡	MC	M.T.	0.0	—	—
J.H.	12/1/71	MC	M.T.	88.3*	H.T.	100.0†
	3/1/72§	MC	M.T.	0.0	—	—
	4/26/72	MC	M.T.	60.2*	J.H.	96.9*

*$P<0.005$.
†$P<0.05$.
‡Death within 1 week of test.
§Rapid spread of disease between 3/1 and 3/18/72. Surgery for partial removal of superficial masses on 3/29/72.

A.J., H.G., and H.R.), or who had partial surgical intervention (J.H.). Of 4 patients who had detectable CMI at an earlier time, 3 became negative when close to death. Neither of the 2 patients who had been positive for SBF remained so during the last week of life. The SBF were assayed with lymphocytes from patients with proven CMI. Thus, both CMI and SBF became negative during the terminal stages of melanoma. Patient J.H. also lost detectable CMI with advancing disease but regained it after surgery.

Another instance of major change in clinical status is surgical removal of a tumor. Previous reports of breast cancer patients (*11*) have shown that CMI may become undetectable within the week after surgery. CMI could not be found in 4 of 8 breast cancer patients during this time, although it was present in 10 of 10 patients who either had surgery >1 week earlier, or had received no surgical treatment. SBF, however, were detectable in 5 of 5 patients within the week post surgery. Although only 2 of our melanoma patients had surgery during the testing period, one of these (J.A.) also lost CMI in the immediate post-surgery period (table 4). Unfortunately SBF were not tested in this patient before or immediately after surgery.

TABLE 4.—*Effect of surgery on CMI and blocking immunity to melanoma cells in patient J.A.**

Date of test	Average No. of cells		Percent significant CMI	Average No. of cells with patient's lymphocytes and		Percent significant SBF
	Control's lymphocytes	Patient's lymphocytes		Control's serum	Patient's serum	
3/8/72	57.3±6.9	29.0±1.4	49.4†	—	—	—
3/21/72‡	24.0±2.6	26.7±7.6	0.0	—	—	—
4/10/72	22.0±2.8	3.0±2.7	81.7†	3.0±2.7	2.0±0.8	0.0

*All tests were by MC assay. Target cell donor was M.T.
†$P<0.005$.
‡Surgery was performed on 3/14/72.

They were not detected, however, 3 weeks after surgery. As far as known, J.A.'s tumor was completely excised.

Tables 5 and 6 present sequential data on 2 patients whose disease underwent obvious change during the short testing period. Patient M.T. (table 5) was first tested for SBF on 8/2/71, when she was thought to be disease free. Her serum completely blocked the CMI of lymphocytes from another melanoma patient. By 11/9/71, her disease had become clinically active and had spread to the draining nodes. At this time, she had positive CMI but tested negative for SBF. By 12/1/71, her disease had become widespread, and her condition continued to deteriorate throughout the period of observations. Notable findings during this time were a positive SBF test on 12/1/71 and a stimulation of tumor cell growth in the presence of M.T.'s lymphocytes on 1/27/72.

Patient V.B. (table 6) was first tested for CMI on 3/8/72. Not only was she negative but also

TABLE 5.—*CMI and blocking immunity to melanoma cells in patient M.T.*

Date of test	Type of test	Target cell donor	Percent significant CMI by M.T.	Immune lymphocyte donor (SBF test)	Percent significant SBF	Remarks
8/2/71	GEL	A.J.	—	J.B.	100*	Thought disease free
11/9/71	MC	BERO	56.1*	M.T.	0.0	Regional active
12/1/71	MC	M.T.	99.9*	H.T.	100†	Widespread
1/27/72	MC	M.T.	−61.1*	—	—	Rapid deterioration
2/7/72	MC	M.T.	79.8*	—	—	—
2/16/72	MC	M.T.	45.9*	—	—	—
3/27/72	MC	M.T.	68.7*	—	—	—

*$P<0.005$.
†$P<0.05$.

TABLE 6.—*CMI and blocking immunity to melanoma cells in patient V.B.**

Date of test	Percent significant CMI by V.B.	Immune lymphocyte donor (SBF test)	Percent significant SBF	Remarks
3/8/72	−15.8†	N.T.	100†	Regional active; large, fungating primary tumor
3/21/72	0.0	N.T.	0.0	
3/29/72	50.2‡	V.B.	100‡	
4/4/72	39.9‡	V.B.	0.0	Early regression of primary tumor
4/25/72	35.0	V.B.	0.0	Continued regression
5/17/72	53.1‡	N.T.	0.0	

*All tests were by MC assay. Target cell donor for all tests was M.T. 3/13–3/17/72, 4/20–4/24/72 } dimethyltriazenoimidazole carboxamide, 250 mg, intravenously, daily.

†$P<0.05$.
‡$P<0.005$.

her lymphocytes significantly stimulated growth of the tumor cells in culture. Tests with lymphocytes from another melanoma patient found her positive for SBF on that date. On 3/21/72, she was negative for both CMI and SBF. On 3/29/72, she tested positive for both factors. Finally on 4/4, 4/25, and 5/17/72, she was positive for CMI and negative for SBF. V.B. was treated daily from 3/13 to 3/17/72 and from 4/20 to 4/24/72 with 250 mg of dimethyltriazenoimidazole carboxamide (DTIC) intravenously. After this treatment, her tumor partially regressed. We are continuing to follow her closely.

DISCUSSION

The data are representative of those from a larger, ongoing study which currently comprises 30 melanoma patients. Although still in its early stage, some conclusions from this work seem justified. Antitumor, CMI, as detected by CI or MC assays, seems to be a regular feature in patients whose disease is remaining relatively stable. It may be lost, however, with rapidly spreading disease, as in patient J.H. (table 3) and is frequently lost within a week of death. Early indications are that CMI may sometimes be depressed by surgery, only to rebound later.

An occasional disturbing observation (*see* patients M.T. and V.B., tables 5 and 6) is increased tumor cell growth in vitro in the presence of the patient's lymphocytes. This observation may be related to "immunostimulation" as envisioned by Prehn and Lappé (*12*). The clinical significance is uncertain.

Contrary to CMI, SBF are not necessarily constant during clinically stable disease. Thus, although patients J.A. and A.T. (table 2) remained free of both detectable SBF and clinically active melanoma, patient I.B. developed SBF without exacerbation of disease. The significance of this is unknown at this time. Patient I.B. may, of course, show signs of disease in the near future, as happened in patient M.T. (table 5). The positive SBF assay may signify a flareup of a focus of melanoma cells successfully destroyed before reaching a clinically detectable level. On the other hand, SBF may fail to be detected even though clinically active disease is known to be present, as in patients M.T. (table 5) and V.B. (table 6). It may also be absent during overwhelming tumor load, as shortly before death. This would suggest that SBF are not soluble, circulating antigens alone. If, as has been suggested, SBF are antigen-antibody complexes, it may be necessary that the 2 reactants be in some fixed proportion which would be upset by either antigen or antibody excess. Alternatively, other serum factors, such as those able to "arm" normal lymphocytes and make them specifically cytotoxic (*13*), may dilute SBF to undetectable levels. In any event, SBF assay may be of value in predicting clinical reaction. Thus SBF was detected 3 months before resurgence of disease in patient M.T. (table 5). Loss of SBF correlated with a good clinical response in V.B. (table 6).

The data obtained so far suggest that the CI and MC assays are a valuable adjunct to management of melanoma patients. However, many

problems with these tests must be solved before they can be widely used. Currently, our most pressing problem concerns the separation of lymphocytes. The plasmagel method is unsatisfactory and frequently results in low yield and nonspecific toxicity. The selection of control lymphocytes can also be a problem. Certain "normal" donors may have lymphocytes able to kill tumor target cells. Control cytotoxicity is rarely, however, as great as that of a patient. It could possibly be overcome by blood group or HL-A type matching of lymphocyte and tumor cell donor, although the necessity to do this may put the test out of reach of all but large medical centers. It is apparent that these methodologic problems require urgent attention before the above types of tests can be generally useful.

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Cytotoxicity of Lymphocytes to Established Cultures of Human Tumors: Evidences for Specificity [1, 2]

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SUMMARY—Established cell lines of human tumors were grown in the wells of LabTek chamber/slide cultures at 37° C in 5% CO_2 atmosphere. The growth rate was expressed as curves by plotting daily cell counts against time. Exposure to buffy-coat leukocytes from patients with tumors corresponding to the target cells rapidly reduced the number of target tumor cells; destruction of target tumor cells by lymphocytes was visible. Established cultures of tumor cells other than those corresponding to the histologic type of the patient's tumor continued to grow in the presence of the patient's leukocytes for several days, but cytotoxic damage to the target tumor cells occurred after several days of cocultivation. It was postulated that patients with tumors circulated lymphocytes that had been presensitized to tumor antigens in vivo and thus interreacted with the corresponding tumor cells promptly after encounter; noncommitted lymphocytes could acquire immune reactivity in vitro but only after several days of cocultivation with the target cells. Lymphocytes activated by tumor cells or by supernatants of tumor cell cultures displayed increased cytotoxicity toward the activator or closely related cultures. Established cultures of lymphocytes were nonspecifically cytotoxic to a battery of target tumor cells. Purified lymphocytes were less cytotoxic than the full buffy-coat preparations. The cytotoxicity of purified lymphocytes was subject to antagonism or synergism by serum factors.—Natl Cancer Inst Monogr 37: 9–18, 1973.

THE IDEAL TEST system for the demonstration of cell-mediated immunity in vitro by cytotoxicity to human tumor cells should consist of 3 basic components: 1) target tumor cells expressing tumor-specific antigens and no stromal cells; 2) lymphoid cell suspensions free of red blood cells, granulocytes, and monocytes; and 3) a medium which is not stimulatory to the lymphocytes, i.e., free of antigens such as bovine proteins or antibiotics that may elicit a blastogenic response in the lymphocytes. The test to be reported herein complies with the first requirement and can be adapted to yield assent to the second requirement. Although problems concerning the third requirement remain unsolved, this and several other tests (*1–5*) indicate that it will be possible to develop an in vitro system for the specific and quantitative measurement of cell-mediated immunity to tumor cells in man and that these tests will also detect serum factors antagonistic to, or synergistic with, the cell-mediated immune reactions. The correlation of these immunologic parameters as measured in vitro with the clinical events occurring in vivo will require scrupulous fur-

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ther studies, for the role of neither the cytotoxic lymphocytes nor the blocking serum factors in the impediment or advancement of tumors has as yet been clearly established (*6*).

MATERIALS AND METHODS

Cytotoxic test.—Fully grown cultures of target tumor cells were rinsed with a 0.02% solution of ethylenediaminetetraacetate (EDTA) and then removed from the glass by a 0.25% solution of commercial trypsin. As the tumor cells were brought into suspension, tissue culture medium was added to dilute the trypsin and the cell suspension was washed with fresh medium by centrifugation in the cold. The tumor cells resuspended in fresh medium were counted, and the count was adjusted so that viable 10^3–$10^{3.7}$ target tumor cells were dispensed into each well of the LabTek chamber/slide culture vessels, each slide carrying 8 chambers. Preparations of leukocytes or purified lymphocytes were added to the appropriate wells. The counts of viable leukocytes were adjusted so that the target tumor cells were outnumbered at least 100:1, preferably 200:1 or more.

To extract leukocytes, heparinized (containing benzyl alcohol as preservative) venous blood was mixed with an equal volume of 6% dextran (National Biochemical) in phosphate-buffered saline. After 45 minutes, the supernatant was decanted and centrifuged, and the buffy coat was centrifuged again in hematocrit tubes; the leukocytes were resuspended in tissue culture medium and counted by the trypan-blue exclusion technique in hemocytometers. Purified lymphocytes were prepared by one of us (K.T.) from the buffy coat of heparinized venous blood without separation of erythrocytes with dextran by the Ficoll-Hypaque technique. To separate all phagocytic cells, including monocytes, from the lymphocytes, carbonyl iron (GAF Corporation) was incubated with the buffy-coat preparations before centrifugation in the Ficoll-Hypaque gradient (*7*). The sediment contained iron-laden granulocytes and monocytes and red blood cells; a layer of highly purified viable lymphocytes was separated above, between the top layer of plasma and the Ficoll-Hypaque mixture forming the bottom layer (*7*).

The medium was Ham's F10 with fresh glutamine added. It was supplemented with 20% heat-inactivated (56° C, 1 hr) fetal calf serum and it contained penicillin G 100 U/ml, streptomycin 125 μg/ml, and neomycin 50 μg/ml.

Sera for pretreatment of target cells were heat inactivated (56° C, 30 min). Sera and target cells, incubated at 37° C for 30 minutes, were transferred into the appropriate wells of the LabTek chamber/slide cultures, and the leukocyte preparations were added; thus, patients' and control sera remained in the wells in excess for the first 3 or 4 days of the experiment.

After 3 or 4 days' incubation, media were withdrawn and replaced by fresh media. Daily, Wright's-stained slides were prepared for inspection and cell counts. Experiments were carried out in duplicate. The target tumor cells adherent to glass were counted in each well in 10 random fields using a 10 × ocular and a 10 × objective. The random fields in which cells were counted were positioned under the microscope so that 5 fields were counted at the edges or in the corners of each well and 5 fields in the center. When occasional large cell clumps were encountered, the right, adjacent field was counted. The experiments were not coded, but the person doing the cell count was not necessarily aware of the contents of the wells. The cell counts were averaged to 1 field and then the 2 values for the duplicates were averaged to 1 value; this latter value was plotted on a graph against time. The 95% confidence levels of the geometric means of the cell counts indicated that twofold differences were significant in most experiments and threefold differences were always highly significant. Values reported in text-figure 4 were calculated by Dr. B. W. Brown, Department of Biomathematics. A comparison of the geometric means indicated that 20% differences between points on 2 curves were already significant.

Target cell lines.—The target tumor cells were: the rhabdomyosarcoma cell line of McAllister et al. (*8*) under protocol #2089, the squamous cell carcinoma of the uterine cervix cell line of Sykes et al. (*9*) under protocol #2043, the Lasfargues-Ozzello cell line of breast carcinoma (*10*) under protocol #2305, and the osteogenic sarcoma cell line of Priori et al. (*11*)

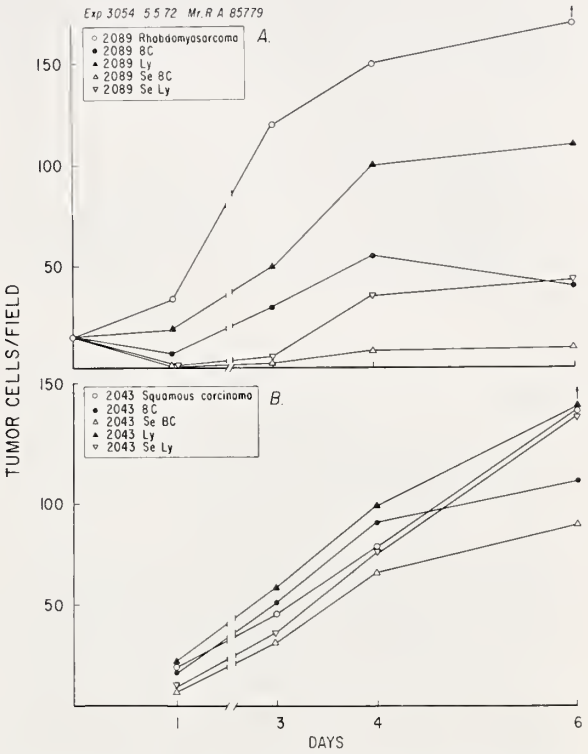
under protocol #1757. Cell lines established in the authors' laboratory were the kidney carcinoma culture (*12*) under protocol #1524, the malignant lymphoma cell line (*13*) under protocol #778, and the malignant melanoma cell line (unpublished) under protocol #2124.

Lymphoid cell lines.—Permanent suspension cultures of lymphoid cells were established in the authors' laboratory. Culture #1401 was derived from the spleen of a woman with squamous cell carcinoma of the uterine cervix who developed idiopathic thrombocytopenic purpura after radiotherapy (*13*). Culture #1806 was derived from a malignant melanoma, culture #2177 from an angiosarcoma, and culture #2460 from a breast carcinoma (unpublished). All cultures reported in this paper have been submitted for further studies to investigators at the National Cancer Institute, Bethesda, Maryland. The cultures were periodically examined by cultural procedures and electron microscopy for mycoplasma contamination. The cultures described in this report were free of such contamination.

RESULTS

Cytotoxic Tests With Patients' Lymphocytes

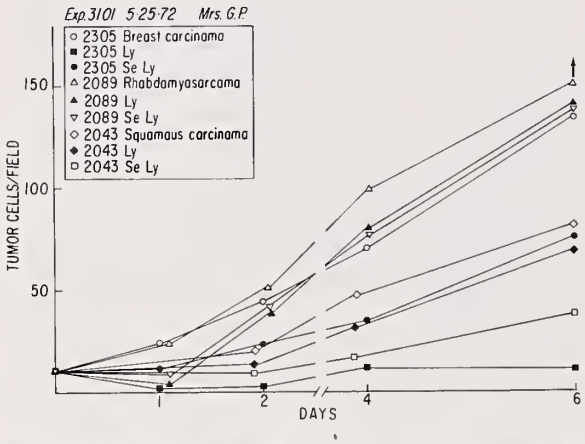
Text-figure 1 shows the growth curve of McAllister's rhabdomyosarcoma cell line (protocol #2089) with and without exposure to buffy-coat preparations or to purified lymphocytes of a young man (Mr. R.A.) with rhabdomyosarcoma of the left thigh. This tumor was excised and followed by extensive radiotherapy and chemotherapy with cyclophosphamide, vincristine, and actinomycin D. At the time of the test, the patient was free of tumors and was receiving no chemotherapy. The growth of the rhabdomyosarcoma cells was strongly inhibited by his buffy-coat preparation and weakly by his purified lymphocytes; actual destruction of tumor cells by lymphocytes was seen (fig. 1). The cytotoxic effect of both buffy-coat leukocytes and purified lymphocytes was potentiated by the patient's heat-inactivated serum. The patient's purified lymphocytes failed to acquire cytotoxic potency to Sykes' squamous cell carcinoma cells (protocol #2043) with or without pretreatment with serum. However, the full buffy-coat preparation showed a weak cytotoxic effect by day 6 that was slightly potentiated by the serum.



TEXT-FIGURE 1.—*A*. Growth of rhabdomyosarcoma culture #2089 was significantly inhibited by the buffy-coat (BC) leukocytes of a patient (Mr. R.A.) with rhabdomyosarcoma. The purified lymphocytes (Ly) weakly inhibited the growth of tumor cells, except for day 3 when the extent of inhibition was highly significant. The serum (Se) of the patient significantly potentiated the cytotoxic effect of both BC leukocytes and Ly. *B*. Neither the BC leukocytes nor Ly of this patient inhibited the growth of squamous cell carcinoma (#2043 cells), except for day 6 when the BC preparation and the BC preparation with Se showed weak inhibition.

Text-figure 2 shows the growth curves of 3 tumor target cell lines: McAllister's rhabdomyosarcoma (protocol #2089), Lasfargues' breast carcinoma (protocol #2305), and Sykes' squamous cell carcinoma (protocol #2043) without and with exposure to full buffy-coat preparations of a woman (Mrs. G.P.) with breast carcinoma metastatic to lungs. There was strong inhibition of the growth of breast carcinoma cells only; treatment of the target cells with the

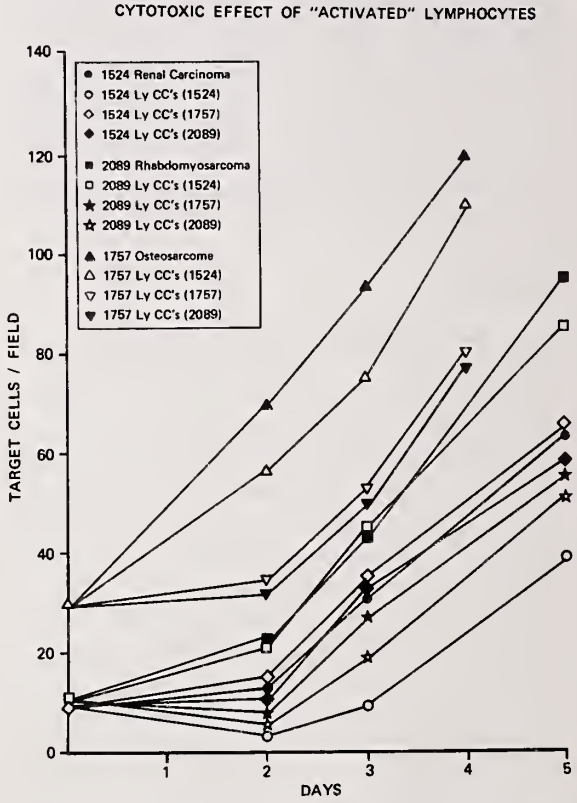
patient's serum before exposure to the buffy-coat leukocytes resulted in significantly less cytotoxicity; i.e., blocking effect was evident. Of the control cells, a slight but significant inhibition of the squamous carcinoma cells occurred on day 6 upon exposure to both serum and leukocytes.



TEXT-FIGURE 2.—Patient, Mrs. G.P., with carcinoma of the breast metastatic to the lungs yielded lymphocytes (Ly) (measured as buffy coat, BC) highly cytotoxic to breast carcinoma cells (#2305) but not cytotoxic to rhabdomyosarcoma (#2089) or squamous carcinoma cells (#2043), except for days 4 and 6 when the serum-pretreated squamous carcinoma cells were inhibited by the BC preparation. The patient's serum (Se) blocked the cytotoxic effect of her lymphocytes on breast carcinoma cells.

Cytotoxic Tests With Activated Lymphocytes

Buffy-coat lymphocytes from a healthy donor (Mrs. C.C.), whose identical twin sister succumbed to rhabdomyosarcoma, were placed on sheets of 3 established tumor cell lines for 24 hours. The leukocytes, retrieved by shaking the cultures, were washed by centrifugation and resuspended in fresh tissue-culture medium. Of the retrieved cells, 80% were viable and 60% were lymphoid and other mononuclear cells. Cytotoxic tests were set up in rubber-stoppered Leighton tube cultures with the 3 target tumor cell lines exposed to each of the 3 leukocyte preparations retrieved from the target tumor cell cultures. Because of the paucity of lymphocytes in these preparations, the target cell:leukocyte ratio in these cultures was set at 1:100, i.e., approximately 60 lymphoid or mononuclear cells:1 target tumor cell. Text-figure 3 shows that lymphoid cells activated on renal carcinoma cells (protocol #1524) significantly inhibited the growth of renal carcinoma cells (inhibition was most significant on day 3) but failed to inhibit the growth of the sarcoma cultures. Contrary to these results, lymphoid cells activated on the 2 sarcoma cultures (protocols #1757 and #2089) inhibited growth of both sarcoma cultures equally well (inhibition of culture #1757 was most significant on days 2 and 3 and that of culture #2089 on 3 and 5) but not that of the renal carcinoma culture. The cytotoxic effect, however, was weak, and all the target tumor cell cultures showed rapid recovery and upward growth curves by day 5.

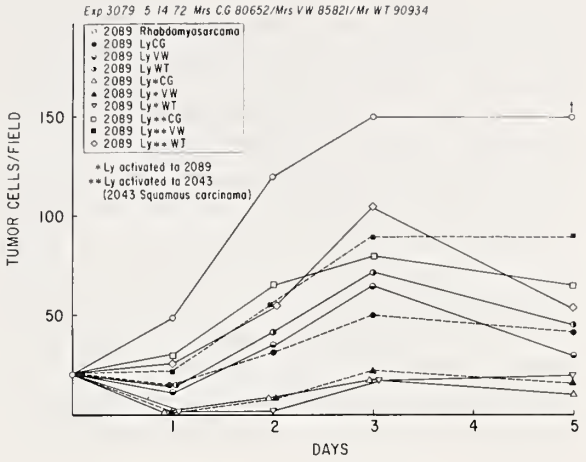


TEXT-FIGURE 3.—Lymphoid cells of a healthy donor (Mrs. C.C.) were activated by exposure to fully grown sheets of renal carcinoma (#1524), rhabdomyosarcoma (#2089), and osteosarcoma (#1757). Lymphoid cells retrieved from the kidney carcinoma culture (Ly CC's 1524) inhibited the growth of kidney carcinoma cells but not that of the sarcoma cultures. Lymphoid cells retrieved from the sarcoma cultures (Ly CC's 1757 and Ly CC's 2089) inhibited the growth of the sarcoma cultures but not that of the kidney carcinoma culture.

Buffy-coat preparations were collected from 3 patients. Mrs. C.G. had chondrosarcoma, Mrs. V.W. had Paget's disease of the bone (osteitis deformans considered to be a presarcomatous lesion), and Mr. W.T. had rhabdomyosarcoma. Samples of the buffy-coat leukocytes were incubated overnight with cell-free supernatant of a 4-day-old, fully grown culture of McAllister's rhabdomyosarcoma cell line (protocol #2089) or of a 5-day-old, fully grown culture of Sykes' squamous cell carcinoma cell line (protocol #2043). The leukocytes were then sedimented by centrifugation and resuspended in fresh tissue-culture medium. These preparations contained 90% viable cells, 50–70% of which were lymphoid or mononuclear cells. The leukocyte preparations were mixed with tumor target cells in a ratio of 100 leukocytes:1 target cell, and the cell mixtures were set in the wells of LabTek chambers/slide cultures.

Text-figure 4 shows that the buffy-coat preparations without prior activation significantly inhibited the growth of the rhabdomyosarcoma target cells. However, an almost complete and sustained inhibition of these target cells occurred after exposure to leukocyte preparations that were preincubated with the supernatant of the rhabdomyosarcoma cell line. The difference between the inhibition of growth by the original buffy-coat preparation and by the pretreated leukocytes was highly significant on days 1, 2, and 3. There was less inhibition after exposure of the rhabdomyosarcoma cells to leukocyte preparations that were preincubated with the supernatant of the squamous cell carcinoma culture. The differences between the extent of inhibition by the original buffy-coat preparations and by the leukocytes pretreated with supernatant of the squamous cell carcinoma culture were of borderline significance on days 1 and 2; on day 3, the buffy-coat preparation of Mrs. C.G. inhibited the growth of the target cells significantly more than that of Mr. W.T. which was pretreated with squamous cell carcinoma supernatant. Attempts were made to test the cytotoxic effect of preparations of these patients on the squamous cell carcinoma cells, but no clear-cut results have as yet been obtained.

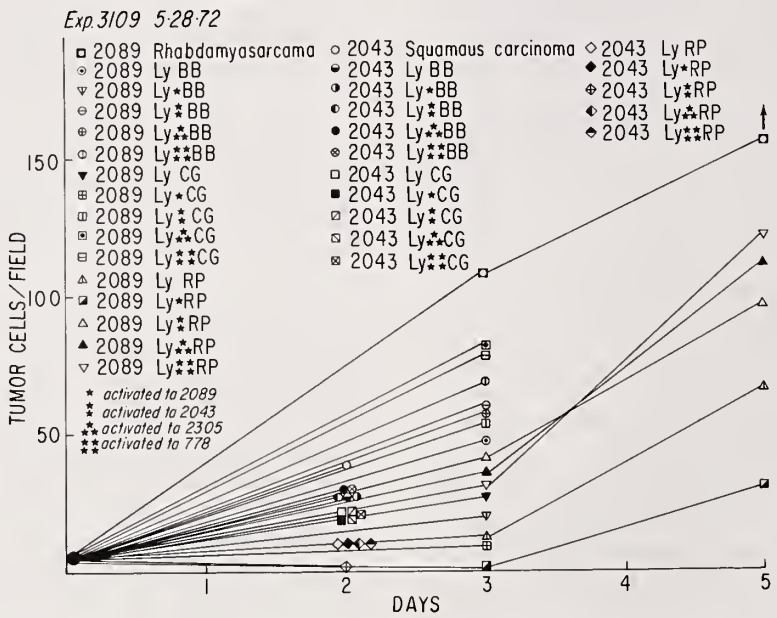
In a similar experiment, the buffy-coat preparations of 3 patients were examined: Mr. B.B.



TEXT-FIGURE 4.—Buffy-coat leukocytes (Ly) of 3 patients with sarcomas were incubated overnight with cell-free supernatants of a rhabdomyosarcoma (#2089) (Ly*) and of a squamous carcinoma (#2043) (Ly**) culture. The retrieved lymphoid cells were tested for cytotoxicity to the rhabdomyosarcoma culture. The lymphoid cells pretreated with the rhabdomyosarcoma supernatant (Ly*) exerted significantly stronger cytotoxic effect than the original buffy-coat preparation (Ly) or than the lymphoid cells pretreated with the squamous carcinoma supernatant (Ly**).

with rhabdomyosarcoma, Mrs. C.G. with chondrosarcoma, and Mrs. R.P. with rhabdomyosarcoma. As in the previous experiment, the patients were receiving no treatment at the time the blood was drawn. Samples of the leukocytes were incubated overnight with cell-free supernatants of fully grown cultures of the following established cell lines: rhabdomyosarcoma (protocol #2089), squamous cell carcinoma of the uterine cervix (protocol #2043), breast carcinoma (protocol #2305), and malignant lymphoma (protocol #778). The leukocytes were then sedimented and resuspended in fresh tissue-culture medium. These preparations contained 80% viable cells, 60–68% of which were lymphoid or mononuclear. The original (not pretreated) buffy-coat preparations and the pretreated leukocyte preparations were mixed with rhabdomyosarcoma (protocol #2089) and squamous carcinoma (protocol #2043) target cells in ratios of 100 leukocytes:1 target cell, and the cell mixtures were set in the wells of LabTek chamber/slide cultures. Text-figure 5 shows that all 3 of the original buffy-coat preparations

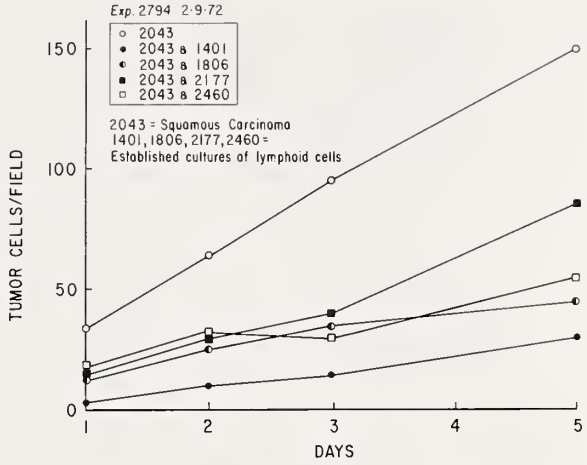
significantly inhibited the growth of the rhabdomyosarcoma cells. Leukocyte preparations preincubated with supernatant of the rhabdomyosarcoma culture displayed significantly greater inhibitory potency than the orginal buffy-coat preparations in all 3 cases. Leukocyte preparations preincubated with supernatants of breast carcinoma, squamous cell carcinoma, or malignant lymphoma cultures inhibited the growth of rhabdomyosarcoma cells less than the original buffy-coat preparations. Leukocyte preparations preincubated with supernatant of the squamous cells carcinoma culture did not show significantly stronger inhibitory potency than other leukocyte preparations except for those of Mrs. R.P. The leukocyte preparation of Mrs. R.P. preincubated with the supernatant of the squamous cell carcinoma culture strongly inhibited the growth of the squamous cell carcinoma culture, whereas other leukocyte preparations of this patient were less inhibitory to these target cells but still more inhibitory than the leukocyte preparations of the other patients tested. In addition to rhabdomyosarcoma, this patient has recently had carcinoma of the uterine cervix in situ. The significance of these tests with the squamous cell carcinoma culture is to be pursued further.



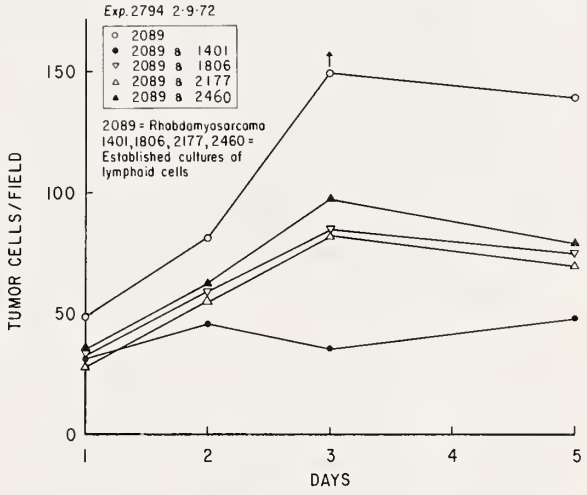
TEXT-FIGURE 5.—Buffy-coat leukocytes of 3 patients with sarcomas were incubated overnight with cell-free supernatants of a rhabdomyosarcoma (#2089), of a squamous carcinoma (#2043), of a breast carcinoma (#2305), and of a malignant lymphoma (#778) culture. The retrieved lymphoid cells were tested for cytotoxicity to the rhabdomyosarcoma and to the squamous carcinoma cultures. The lymphoid cells pretreated with the rhabdomyosarcoma supernatant (Ly*) exerted stronger cytotoxic effect than the lymphoid cells pretreated with squamous carcinoma (Ly⁂), breast carcinoma (Ly⁂), or malignant lymphoma (Ly⁂) culture supernatants or the original buffy-coat (Ly) preparation. All lymphocyte preparations weakly inhibited (no or borderline significance) the squamous carcinoma culture except that of Mrs. R.P. The lymphocyte preparations of this patient exerted the strongest inhibition on squamous carcinoma cells, and all growth was completely inhibited by the lymphocyte preparation preincubated with squamous carcinoma culture supernatant (Ly⁂).

Cytotoxic Tests With Established Cultures of Lymphoid Cells

When suspension cultures of lymphoid cells from patients with lymphoreticular neoplasia were tested against various target tumor cells, a "broad spectrum," nonspecific, cytotoxic effect was observed which could not be blocked by tumor-specific blocking factors of patients' sera (*14*). Cross-cytotoxic tests were prepared to determine the degree of cytotoxicity of established cultures of lymphoid cells to target tumor cells derived from the same pathologic entities as the lymphocyte cultures. Established suspension cultures of lymphoid cells from squamous cell carcinoma of the uterine cervix and idiopathic thrombocytopenic purpura (protocol #1401), from malignant melanoma (protocol #1806), from angiosarcoma (protocol #2177), and from breast carcinoma (protocol #2460) were tested against the established allogeneic tumor cell cultures of squamous cell carcinoma of the uterine cervix (protocol #2043), malignant melanoma (protocol #2124), rhabdomyosarcoma (protocol #2089), and breast carcinoma (protocol #2305). Although the ratio of target tumor cells and lymphocytes was set at the beginning of these experiments as 1:100, the rapid replication of the lymphocytes later shifted this ratio more to the favor of the lymphocytes. To prevent target cell inhibition due to competition for nutrients, these cultures were given fresh medium on the 3d day of incubation. Text-figures 6 through 9 show that there was little, if any, specific correlation between the pathologic entity from which established cultures of the lymphocytes derived and the type of target cells against which the lymphocytes were tested.



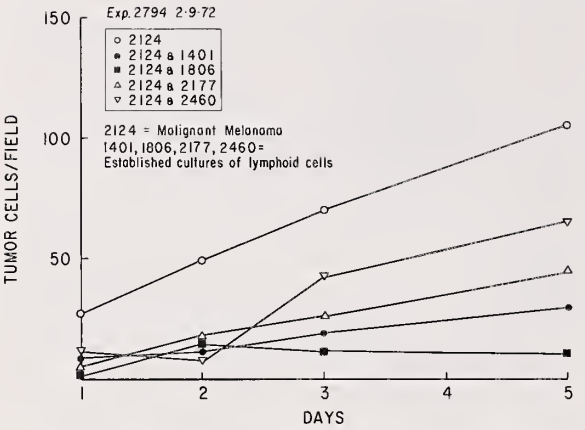
TEXT-FIGURE 6.—Established cultures of lymphoid cells (#1401, #1806, #2177, and #2460) inhibit the growth of squamous carcinoma cells (#2043).



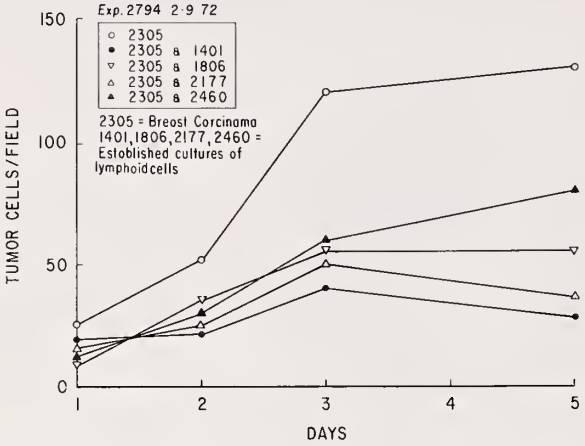
TEXT-FIGURE 7.—Established cultures of lymphoid cells (#1401, #1806, #2177, and #2460) inhibit the growth of rhabdomyosarcoma cells (#2089).

DISCUSSION

Admittedly, an autologous system utilizing the tumor cells, lymphocytes, and sera of a patient for the detection of cell-mediated immunity in vitro would surpass in specificity the allogeneic systems. In the allogeneic system, however, it was possible to distinguish between lymphoid cells reacting with tumor-specific antigens and those reacting with histocompatibility antigens. When lymphoid cells of patients with rhabdomyosarcoma and of normal individuals were activated by exposure to cultured rhabdomyosarcoma cells, both types of lymphocyte preparations displayed cytotoxic potency to the activator cells. However, pretreatment of the target rhabdomyosarcoma cells with serum containing sarcoma-specific blocking factors resulted in good protection of the target rhabdomyosarcoma cells against the activated lymphocytes of the patients but not against those of the normal individuals (*5, 14*). The most plausible inter-



TEXT-FIGURE 8.—Established cultures of lymphoid cells (#1401, #1806, #2177, and #2460) inhibit the growth of malignant melanoma cells (#2124).



TEXT-FIGURE 9.—Established cultures of lymphoid cells (#1401, #1806, #2177, and #2460) inhibit the growth of breast carcinoma cells (#2305).

pretation of this finding is that the blocking serum factors are tumor antigen-specific but not transplantation antigen-specific. Coating the tumor antigens but not the transplantation antigens on the cell surface by these serum factors prevents the access of lymphocytes committed to tumor antigens but not that of lymphocytes committed to transplantation antigens. Patients with sarcomas circulate a lymphocyte population that has undergone presensitization to sarcoma-specific antigens in vivo; the progeny of these specifically cytotoxic cells thus can be inhibited by serum factors specific to sarcoma antigens. Noncommitted lymphocytes of normal individuals acquire, in the presence of blood monocytes, de novo cytotoxic potency to foreign cells; however, most of the lymphocytes so activated are directed to foreign histocompatibility antigens and not to tumor-specific antigens. Thus tumor antigen-specific serum factors cannot block the access of these lymphocytes to the target cells (*14*). Tumor antigen-specific serum factors have provided indirect evidence for the tumor-distinctive specificity of patients' lymphocytes in the cytotoxic test. Further evidence for the tumor-distinctive specificity of patients' lymphocytes is the rapid destruction of tumor target cells corresponding to the histologic type of the patient's tumor as opposed to the late-appearing cytotoxic effect directed toward unrelated target cells which appears in the end phase of the reaction. The former effect is thought to be mediated by lymphocytes presensitized in vivo to tumor-specific antigens, whereas the latter is attributed to an immune reaction newly acquired in vitro. The effect of presensitized lymphocytes is presumably tumor specific even in allogeneic systems, whereas the immune reaction acquired in vitro appears to be directed primarily to histocompatibility antigens.

Exceptions to this generalization have been observed in the authors' laboratory frequently enough to warrant the following further tests: 1) using large numbers of normal buffy-coat preparations against a battery of target cells to determine whether the rapid reaction pattern does necessarily indicate the activity of lymphocytes presensitized in vivo or whether immune reactivity may be newly acquired very rapidly in vitro; 2) comparing the cytotoxic effects of purified lymphocyte as opposed to full buffy-coat preparations of the same donors against the same target cells; 3) using throughout the series of related experiments samples of target cells stored deep-frozen (these target cells would be shown to express tumor antigens); 4) attempting to interreact lymphocytes and target cells in a serum-free environment; and 5) providing greater precision through better instrumentation (for this, the introduction of automatic micropipetting and and automatic cell counting will be necessary). The exceptions to the generalizations outlined above were: 1) the rapid destruction of tumor cell cultures by the

lymphocytes (or buffy-coat preparations) of a few normal (laboratory and hospital personnel) individuals (*5*); 2) the resistance gained by tumor cells to cytotoxic lymphocytes in vitro (*5*); 3) the growth-promoting effect of certain lymphocyte or serum preparations on certain tumor cell lines (particularly, squamous carcinoma line #2043); 4) the acquisition of cytotoxic potency by buffy-coat preparations to tumor cell lines that were histologically unrelated to the patient's tumor with the introduction of the patient's serum into the system; and 5) the nonspecific antilymphocyte effect of certain patient's sera (*14*) (such sera could incapacitate cytotoxic and noncommitted lymphocytes). These observations suggest that the present systems for the in vitro monitoring of immune reactions of patients to tumors are complicated and should not, at this time, be accepted in an oversimplified form, i.e., that the destruction of tumor cells by patients' lymphocytes always represents a unique reaction which is not expressed by lymphocytes of normal, unrelated individuals and that blocking serum factors are always present when tumors advance, implying that these factors necessarily promote tumor growth in vivo.

The present study adds a new argument in favor of the specificity of the cytotoxic test. The fact that activated lymphocytes displayed significantly stronger cytotoxic effect toward the activator cell line than toward unrelated cell lines confirms recent experiments in which specific sensitization of human peripheral blood leukocytes to established lymphoid cell lines was shown (*15*). When established lymphoid cell lines were tested for cytotoxic effect on various target cells, no such specificity could be demonstrated. This finding is in accord with the concept that lymphoblastoid cells manufacture and release cytotoxins in an "autonomous" and nonspecific fashion (*12, 16*).

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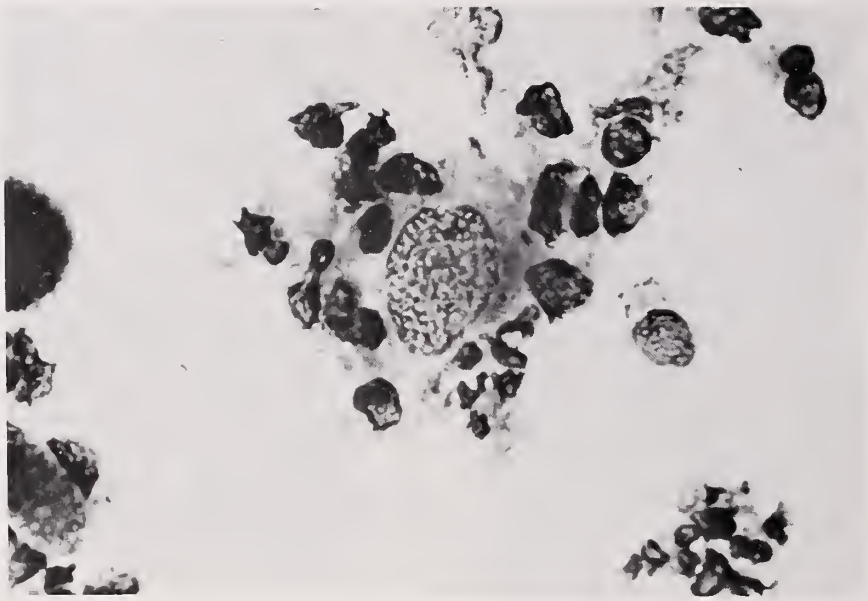

FIGURE 1.—Lymphoid cells of patient (Mr. R.A.) with rhabdomyosarcoma surround degenerating cultured allogeneic rhabdomyosarcoma cell. Oil immersion. Wright's stain. × 540

Standardization of Microcytotoxicity Assay for Cell-Mediated Immunity [1, 2]

Carol O'Toole,[3] *Department of Immunology, Wenner Gren Institute, Norrtullsgatan 16, Stockholm 113 45, Sweden*

SUMMARY—The microcytotoxicity assay of Takasugi and Klein gives a reproducible and quantitative estimation of specific cell-mediated tumor immunity in human urinary-bladder carcinoma. Quantitation requires titration of the effector-to-target-cell ratio, with effector cell preparations from patients and controls of equivalent purity. Results can be reproduced with a cell line derived from the relevant tumor type used as the reference target cell. The method of effector cell purification used routinely has given reproducible, specific results. A standard reference effector cell may be obtained from purified cytotoxic lymphocytes stored at —130° C. Nonspecific effects in the assay have been observed with effector cell preparations containing granulocytes or eosinophils at levels >10%. The method of lymphocyte purification may also nonspecifically influence target cell survival, as seen after use of Ficoll-Isopaque gradient separation. The assay described here was used to quantitate cellular immunity to carcinoma of the bladder in patients of varying clinical conditions. Cell-mediated immunity appears to depend on the presence of critical amounts of viable tumor or tumor-derived material in the body, while it may be inhibited by excessive quantities of either.—Natl Cancer Inst Monogr 37: 19–24, 1973.

TO ASSESS the role of therapy and clinical course of the disease on the tumor-directed immune response, standardized and quantitative techniques are required. Studies on cell-mediated immunity to neoplasia in man have relied mainly on the colony-inhibition test (*1*) or the microcytotoxicity assay (*2*). In general, these methods have shown that tumors of the same histogenic origin have common antigenicity (*3–5*). This has facilitated the use of allogeneic tumor cell lines as the standard target material in the cytotoxicity assay. Reproducibility in the system can be achieved when lymphocyte preparations are of equivalent purity. Under these conditions, the specific cell-mediated response to tumor can be quantitated and shown to relate to the clinical condition of the patient (*6, 7*).

MATERIALS AND METHODS

Blood samples (about 30 ml) were taken either from patients with carcinoma of the urinary bladder or other neoplastic diseases or from healthy donors. Each donor was tested on several occasions. Carcinoma of the urinary bladder was staged according to UICC classification (*8*).

Preparation of effector cells.—Lymphocytes were obtained from defibrinated blood. The standard technique involved sedimentation of the erythrocytes through gelatin (*9*); adherent cells were then removed by incubation of the leukocyte preparation on a nylon wool column (*10*). Finally, remaining erythrocytes were lysed by Tris ammonium chloride (*11*). The cells were then washed 3 times in Tris-Hanks' solution containing 2.5% fetal bovine serum.

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[1] Presented at Conference and Workshop on Cellular Immune Reactions to Human Tumor-Associated Antigens, held at the National Cancer Institute, June 8 and 9, 1972.

[2] This work was supported by grant No. 365-K72-03XB from the Swedish Cancer Society.

[3] I gratefully acknowledge the skillful technical assistance of Mrs. Margareta Karlsson.

Alternatively, lymphocytes were obtained by Ficoll-Isopaque gradient separation (*12*). The leukocyte suspension obtained after gelatin sedimentation was layered above a gradient consisting of aqueous solutions of 10 parts 33.9% Isopaque (Nyegård & Co., Oslo, Norway) and 24 parts 9% Ficoll (Pharmacia, Uppsala, Sweden). The gradient was then spun at 400 × *g* for 40 minutes, and the lymphocyte band harvested. The lymphocytes were then washed 3 times. Both methods of purification normally yielded lymphocyte preparations of $\geq$98% purity. All lymphocyte preparations were checked miscroscopically after being stained with May-Grünwald-Giemsa.

Storage of effector cells.—Purified lymphocytes prepared by the standard method were placed in tissue culture medium 199 containing 15% human AB+ serum and 10% dimethyl sulfoxide at a cell concentration of 10×10^6/ml. The preparation was cooled on ice for 1 hour and then frozen at a constant rate of 1°/minute to −50° C. Thereafter it was kept at −130° C in liquid nitrogen. Before being tested, frozen lymphocytes were thawed rapidly to 37° C and then washed 3 times in Tris-Hanks' solution containing 15% fetal bovine serum (FBS).

Incubation of effector cells.—Purified lymphocytes were incubated at 37° C in humidified air plus 5% CO_2 for up to 3 days. The incubation medium was 199 containing 15% human AB+ serum, antibiotics, and glutamine. AB+ serum was more satisfactory than FBS for cell viability. FBS produced blastogenesis in some donors' lymphocytes after a 3-day incubation period (*13*).

Target cells.—These were grown in monolayer cultures. Explants were prepared from fresh biopsy specimens of tumor or normal tissues. All cells were passed by treatment with 0.25% trypsin. To eliminate leukocytic contamination, primary cultures were passaged at least twice before being used as target cells. They were obtained from a transitional cell carcinoma of the urinary bladder, Tu 7, from the stroma of a retroperitoneal lymph node, MYR, and from embryonic lung. The cell line T 24, from a transitional cell carcinoma of the bladder, was the reference target cell. It was tested routinely in parallel with primary cultures of the same tumor type to control possible changes in antigenicity due to long-term culture.

Microcytotoxicity assay.—This was done as described originally by Takasugi and Klein (*2*). The tissue culture medium used in all the tests was 199 containing either 10% human AB+ serum or FBS, antibiotics (100 IU penicillin and 100μg streptomycin/ml), and glutamine 0.3 mg/ml. All sera were inactived before use, AB+ for 30 minutes and FBS for 60 minutes. Thrice-washed target cell suspensions prepared by trypsinization were added to microplates (Falcon 3034) to give 20–50 cells/well. The plates were incubated at 37° C in humidified air plus 5% CO_2 for 3–18 hours to allow cell attachment. Purified lymphocyte preparations were then added. Each donor's lymphocytes were usually tested at $\geq$3 ratios to the target cells. Effector cells from a healthy donor and/or a clinical control were included in each experiment. In addition to those from bladder carcinoma, target cells from other tissues were also tested in each experiment. A minimum of 12 wells was used on every target tissue for each donor's lymphocytes at a given ratio. Target cell viability was checked on every plate in wells receiving only medium. After the plates were incubated for 2 or 3 days at 37° C in humidified air plus 5% CO_2, the lymphocytes were removed by being washed with phosphate-buffered saline. The remaining adherent cells were stained with May-Grünwald-Giemsa.

Evaluation of microcytotoxicity assay.—The average number of residual target cells in wells which had contained patients' lymphocytes was compared with that in wells which had contained control lymphocytes at an equivalent ratio. The significance of the difference between the 2 was calculated by the Student's *t* test at $P\leq0.05$. Cytotoxicity was expressed as the percent reduction in target cell number produced by patients' lymphocytes as compared with that produced by the control donors' lymphocytes (= 100) on each of the target cells tested in a given experiment.

RESULTS

Specific cell-mediated immunity to carcinoma of the bladder was demonstrated repeatedly in

patients in the following clinical situations: in the presence of untreated, small, localized primary tumors stage T1–T2 and after local irradiation to such tumors. During radiotherapy, no such responses were detectable after doses of >1000 R (*6, 7*). As table 1 shows, patients could develop cytotoxic lymphocytes within 3 days after cessation of therapy. This response was maintained without any detectable residual tumor over an 11-month observation period in patient F.P. The results of a larger study on such patients were published (*6*). Table 1 demonstrates the reproducibility of the test system in a given clinical situation. Control lymphocytes tested in parallel with lymphocytes from patient F.P. did not specifically destroy the target cells, even when the donor had received radiotherapy, C11, C46, and C48.

TABLE 1.—*Reproducibility of microcytoxicity test**

Date of test	E:T†	Lymphocytes		Reduction‡ (%)
		Control	Patient's	
		Patient F.P. T2 M2–3§		
10/19/70	500:1	15±8[5]‖	1±1	93
	250:1	22±10	3±3	86
4/29/71	500:1	47±11[6]‖	0	100
	250:1	79±18	2±2	98
	125:1	91±11	4±5	96
5/24/71	500:1	34±18[7]‖	3±4	91
	250:1	84±17	13±7	85
	125:1	71±9	33±15	54
8/31/71	500:1	69±17[8]‖	1±2	99
	250:1	104±21	4±4	96
	125:1	97±20	17±9	82
	63:1	100±16	28±9	72
9/8/71	500:1	86±20[9]‖	0	100
	250:1	105±21	1±2	99
	125:1	110±14	8±2	93
	63:1	100±15	25±11	75

*Numbers are means ± SD of surviving target cells/well after 3 days' incubation with lymphocytes.

†Effector : target cell ratios. Target cells, T 24.

‡Cytotoxicity estimated as percent reduction in surviving target cells after incubation with patient's lymphocytes (number surviving after exposure to control lymphocytes = 100). Significance calculated by Student's t test, $P<0.001$.

§Patient given radiotherapy, treatment ended 10/16/70, 6500 R tumor dose.

‖5, C11 (cancer of prostatae); 6, C46 (hypernephroma); 7, C48 (cancer of prostatae); 8, C66 (cancer of prostatae); and 9, C66 A (healthy control).

Sources of Variation in Assay

Eosinophilia is often observed in patients with large infiltrating tumors, both before and after radiotherapy. The standard method of lymphocyte purification used here in fact enriched the eosinophil component of the blood. Effector cell preparations containing ≥10% eosinophils could nonspecifically destroy all target cell types. This is indicated in table 2 in the patient J9, who had a metastatic tumor of the prostatae. His lymphocytes when "purified" contained 18% eosinophils and significantly destroyed both target cell types tested. In the same experiment, purified lymphocytes from patient J11 (who had a recurrent, localized bladder carcinoma) were specifically cytotoxic for bladder tumor cells. Patient J10 had no evidence of original bladder tumor, and his lymphocytes were not cytotoxic to either target cell. These results, in relation to the clinical situation of the patient, are similar to those published in (*6, 7*).

As described previously (*14*), similar nonspecific cytotoxic effects are seen when effector cell preparations contain >10% granulocytic cells. If the cell-mediated response of the patient is to be quantitated and related to a changing clinical situation, the composition of the effector cell preparations must be known; in those prep-

TABLE 2.—*Influence of eosinophils in effector cell preparation on target cell survival*

Effector cells	Target cells,* T 24 E:T†	500:1	250:1	Fibroblasts, MYR‡ 500:1	250:1	Eosinophils in effector cells (%)
J9§		98	85	61	35	18
J10‖		0	0	0	0	1
J11¶		83	41	0	0	1

*Numbers are percent reduction in surviving target cells after 2 days' incubation with test lymphocytes (number surviving after exposure to control lymphocytes = 100). Significance calculated by Student's t test, $P<0.001$.

†Effector : target cell ratios.

‡Control target cells MYR; fibroblasts derived from culture of retroperitoneal lymph node (second passage).

§Patient with carcinoma of prostatae with metastases.

‖Patient given radiotherapy for carcinoma of the bladder in 1964, tumor in 1972.

¶Patient, given radiotherapy for carcinoma of the bladder in 1969, had recurrent tumor present at test in 1972.

arations containing eosinophils or granulocytes at the levels described, cytotoxicity cannot be definitely quantitated.

The standard technique for preparation of effector cells is time-consuming, although it does give reproducible results with preparations of equivalent purity. The more rapid Ficoll-Isopaque method was therefore tried. Text-figure 1 shows the results obtained with a healthy donor's lymphocytes purified by the 2 methods. Target cell survival was always highest with lymphocytes prepared in the standard way, irrespective of the target cell type. A normal donor's lymphocytes prepared by the Ficoll-Isopaque method significantly reduced target cell survival as compared with the effect produced by the same lymphocytes prepared by the nylon wool-Tris-ammonium chloride method. The reason for this is unclear; all methods shown in text-figure 1 yielded lymphocyte preparations of $\geq 98\%$ purity. However, microscopic observation revealed greater adhesion of lymphocytes prepared by the Ficoll-Isopaque method to the target cells.

To explore the test system further, known positive effector cells are needed. The results obtained with lymphocytes stored in liquid nitrogen are encouraging. Purified lymphocyte preparations must be used for freezing to retain specificity in the cytotoxicity test. As shown in table 3, where the same 2 donors' lymphocytes were tested both before and after freezing, the specific cytotoxicity measured remained quantitatively the same.

Preincubation of purified lymphocytes for up to 3 days before the test does not diminish their cytotoxic potential. Provided that human AB+ serum is used in the incubation medium, no blastogenesis occurs and no nonspecific cytotoxicity results. As seen in table 3, the patient's lymphocytes remained cytotoxic after 3 days' preincubation while the control lymphocytes remained inactive.

TABLE 3.—*Reactivity of effector cells after freeing or preincubation**

Effector cells	E:T†	Lymphocytes: Control	Lymphocytes: Patient's	Reduction‡ (%)	P
Fresh	500:1	69±18§	0‖	100	<0.001
	250:1	93±19	12±8	87	<0.001
	500:1¶	19±10	8±3	58	<0.01
After 10 days' storage at −130° C	250:1	29±11	4±3	86	<0.001
	125:1	28±10	12±5	57	<0.001
3 days' preincubation at 37° C	500:1	60±11	11±5	82	<0.001
	500:1¶	19±8	1±2	95	<0.01

*Numbers are means ± SD of surviving target cells/well after 3 days' incubation with lymphocytes.

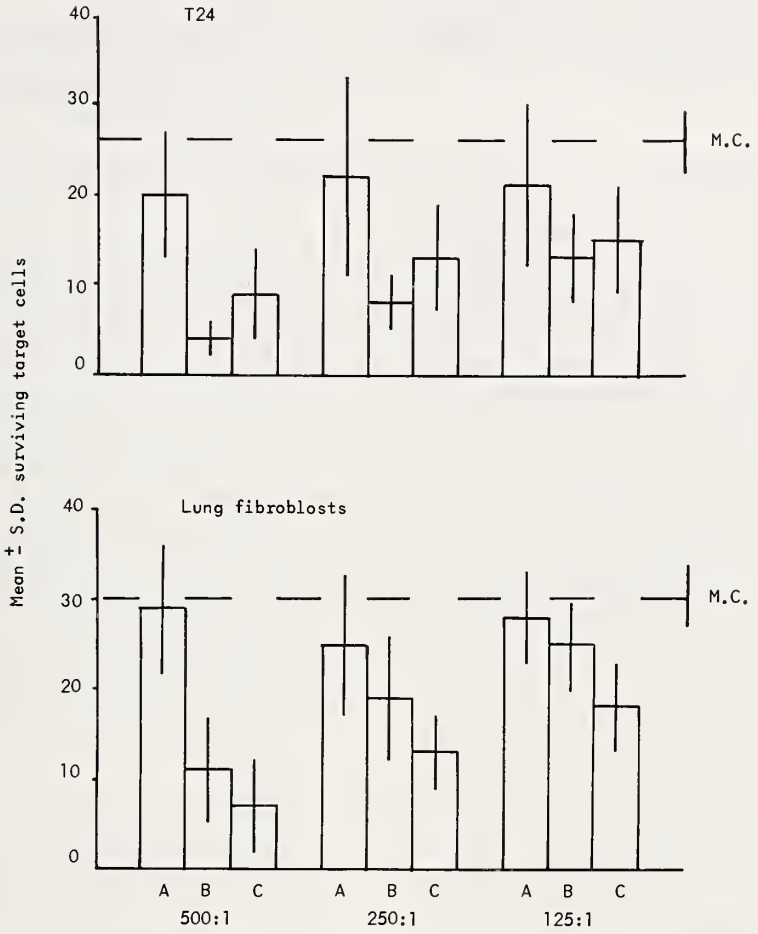
†Effector:target cell ratios. Target cells were T 24 except when indicated.

‡Cytotoxicity estimated as percent reduction in surviving target cells after incubation with patient's lymphocytes (number surviving after exposure to control lymphocytes = 100). Significance calculated by Student's t test.

§Control lymphocytes from healthy donor.

‖Lymphocytes from patient with carcinoma of the bladder, T1–M2.

¶Target cells from primary culture of bladder carcinoma (second passage), Tu 7.



TEXT-FIGURE 1.—Target cell survival in relation to lymphocyte (from healthy individual) purification method. Method A: nylon wool—Tris ammonium chloride; method B: nylon wool—Ficoll-Isopaque; and method C: Ficoll-Isopaque. M.C.: target cells incubated with medium only.

Target		Effector:target ratio	Reduction (%)	P*
T 24	B:A	500:1	80	<0.001
		250:1	64	<0.001
		125:1	38	<0.001
	C:A	500:1	55	<0.001
		250:1	41	0.01
		125:1	29	<0.05
Lung fibroblasts	B:A	500:1	62	<0.001
		250:1	24	**0.02**
		125:1	11	0.1
	C:A	500:1	76	<0.001
		250:1	48	<0.001
		125:1	36	<0.001

*Significance of differences of methods B and C from method A calculated by Student's t test.

Human AB+ serum was used routinely in the microcytotoxicity assay; however, FBS also gave reproducible results, if the assay was terminated at 3 days' incubation. After longer periods, nonspecific cytotoxicity was observed with some donors' lymphocytes that were stimulated to blastogenesis by the FBS (*13*).

DISCUSSION

This test system offers a reproducible assay for cell-mediated immunity to human tumor. Not many effector cells are required for each test; this is of particular importance when irradiated patients are tested, since lymphopenia is common during and after radiotherapy (*15*). The use of cell lines as reference target material has facilitated standardization of the assay. To date, no significant differences have been observed between specific cytotoxicity measured on primary cultures and cell lines derived from the same tumor type. However, most of the tests were performed in an allogeneic system, and the autologous situation requires further study in this respect.

The assay can be reproduced if effector cell preparations are of equivalent purity; contaminating eosinophils or granulocytes can reduce the specificity of the reaction. Patients with eosinophilia or granulocytosis present problems in this test system; although specific reactions may be detectable by their lymphocytes, the response is difficult to quantitate. Thus differential counts must be performed on all "purified" effector cell preparations before being tested. The results obtained with lymphocytes purified by the Ficoll-Isopaque method reveal that the microassay is particularly sensitive to nonspecific effects such as changes in adhesiveness of the effector cells. Before more rapid methods of lymphocyte purification are applied to the assay, the effector cells must be tested on several types of target tissue.

The retention of specific cytotoxicity after freezing offers a realistic method for standardization of the target tissues under test and as a control for day-to-day technical variation. Further flexibility in the system is also gained by the possibility of preincubating the effector cell preparations.

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Stimulation of Lymphocytes In Vitro to Reactions Against Cultured Burkitt's Lymphoma Cells [1, 2]

Sidney H. Golub,[3] John F. Hewetson,[4] Erik A. J. Svedmyr,[5] George Klein,[5] *and* Surjit Singh[6]

SUMMARY—A modified colony-inhibition (CI) test was developed to study cell-mediated reactions against cultured lymphoblastoid cells. Lymphoblastoid culture lines (LCL's) will grow in discrete colonies in semisolid media, where normal peripheral blood lymphocytes cannot. Therefore, surviving target cells in mixtures of lymphocytes and target LCL cells can be distinguished. Immunocompetent cells from patients with Burkitt's lymphoma (BL) were stimulated in mixed leukocyte cultures (MLC) by cocultivation with autochthonous LCL cells treated with mitomycin C. Stimulation was assayed by increased incorporation of tritiated thymidine. After stimulation in MLC, lymphocytes from BL patients showed markedly increased activity in the CI test against autochthonous target cells. CI activity was greatest against autochthonous and allogeneic LCL cells of BL origin and was considerably less against target cells of non-BL origin. Lymph node cells also could be stimulated by these means, although they did, on occasion, show more CI activity before stimulation than did peripheral blood leukocytes. Lymphocytes could not be sensitized with autochthonous fibroblasts treated with mitomycin C. These results indicate the presence of antigens on LCL cells of BL origin that can lead to cell-mediated cytotoxic reactions.—Natl Cancer Inst Monogr 37: 25–32, 1973.

ONE MEANS of demonstrating antigenic disparity between tumor cells and normal lymphoid cells is the mixed-lymphocyte-tumor-cell assay. In this assay, antigenic differences can be recognized by increased DNA synthesis of the lymphocytes. The presence of neoantigens on leukemic cells has been demonstrated with the use of this approach (*1–3*). Similar approaches were reported in a variety of solid tumors, and, although results were positive, the stimulation of autochthonous lymphocytes by tumor cells was usually considerably more difficult to demonstrate than was the case when lymphoid cells were used as the stimulators (*4–7*).

The strongest reactions of this type have been described with the use of cultured lymphoblastoid cells. Highly effective stimulation in autochthonous systems was reported with the use of lymphoblastoid cell lines obtained from infectious mononucleosis (*8, 9*), and also in a number of cases with the use of lymphoblastoid cell lines developed from normal donors (*10–13*). Whether stimulation in these latter cases is due to the presence of Epstein-Barr virus (EBV)-associated antigens, or some other factors, has not yet been established.

Other than its use for the demonstration of antigenic disparities, mixed leukocyte culture

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(MLC) can be used to generate effector cells for cytotoxic tests. Effector cells from MLC are known to have specific cytotoxic activities, both in animal model systems (*14–16*) and with the use of human peripheral blood lymphocytes (*17*).

Previously we (*18–20*) reported a system to study cellular reactions against cultured Burkitt's lymphoma (BL) cells. We showed that effector cells from peripheral blood of BL patients had minimal activity in a colony-inhibition (CI) test against cultured BL cells, although some lymph node lymphocytes had considerably more activity. Despite the apparent lack of cytotoxic activity in the samples studied, leukocytes from BL patients could be routinely stimulated with autochthonous cells from lymphoblastoid culture lines (LCL's). Studies on the cytotoxicity of effector cells stimulated in vitro with autochthonous or allogeneic cells led to the conclusions that antigens on BL-derived LCL cells are cross-reactive and can stimulate leukocytes to yield a cytotoxic response. This study extends those findings.

MATERIALS AND METHODS

Patients.—Although a number of patients were studied in the autochthonous stimulation system, in this report we will concentrate on an extensive study of 2 patients with histologically confirmed BL. These patients, from the Kenyatta National Hospital, were PM (Penina Mukoya, KCC 1140) and SN (Sulubu Ngonio, KCC 1227). PM, an 8-year-old female, presented with BL of the right maxilla and a very large ovarian tumor. Treatment with combined chemotherapy resulted in a complete tumor remission. Following chemotherapy, PM was given 10 vaccinations with Glaxso BCG administered with a Heaf gun as nonspecific adjuvant immunotherapy. To date, she is free of tumor and in good health. SN, an 8-year-old male, originally presented with BL of the mandible and maxilla. Combination chemotherapy induced a remission, which however, was followed by a tumor recurrence a few months later. A second course of chemotherapy induced another remission, but 3 months later the tumor recurred in the thigh. The patient subsequently died.

An LCL was established in culture from a tumor biopsy of patient PM by Dr. Guy de-Thé of Lyon, France, and termed LY-46. In this study, this line will be designated PM-T (patient PM, tumor-derived line), and peripheral blood leukocytes from patient PM will be termed PM-L. A line of normal skin fibroblasts also was established from PM. Several lines from tumor biopsies from patient SN were established by Dr. Balwant Gothoskar of the Karolinska Institute. The first of these lines, designated SN-T, was used in this study along with a line of fibroblasts established from a normal lymph node biopsy. Peripheral blood leukocytes from patient SN will be designated SN-L. Both PM-T and SN-T displayed the membrane and viral capsid antigen associated with EBV in immunofluorescence tests. Two cell lines which do not display these EBV-associated antigens, NC-37 and PE-SS, were used as control cultures. All cultures were maintained in RPMI tissue-culture media supplemented with 20% fetal bovine serum and antibiotics and cultured at 37° C in a humidified, 5% CO_2, incubator.

Mixed cell cultures of peripheral blood leukocytes and LCL cells, instituted in a manner comparable to standard MLC, were established as described previously (*19*). Incorporation of ^{3}H-thymidine is expressed as a response index derived by the formula:

$$\text{Response index} = \frac{\text{counts per minute (cpm) in test culture}}{\text{cpm in control culture.}}$$

Control cultures consisting of peripheral blood leukocytes plus autochthonous leukocytes treated with mitomycin C were included in each experiment.

CI tests.—The CI test of Hewetson et al. (*18*) was used in this study. Briefly, 0.1 ml of target LCL cells and 0.1 ml of effector cells were mixed in the wells of a UV-sterilized, conical bottom, hemagglutination plate (Linbro Chemical Co., New Haven, Conn.). Target cells were added at the concentration of 1×10^4/ml and effector cells in ratios ranging from 100:1 to 3:1 to the target cells. The plate was incubated for 24 hours. Samples from each well were mixed 3 times with a 1-ml syringe fitted to a Hamilton adaptor (Hamilton Co., Whittier, Calif.), and 0.02 ml was dispensed from each well into the corresponding well of a flat-bottom Falcon tissue cul-

ture II microplate (Falcon Plastics, Oxnard, Calif.). An additional 0.02 ml of human AB plasma was added to each well, and the plate was gently tapped to spread the contents of each well evenly. The plasma and media formed a viscous layer that could be overlaid with 0.2 ml tissue-culture media without disturbing cells. The plates then were incubated for 5–7 days until discrete colonies could be visualized on a Leitz inverted microscope at 25 ×. Typical colonies are shown in figure 1, although these colonies were allowed to grow somewhat past the 20–40 cell stage routinely used in the test. Effector cells alone did not form colonies. Thus the number of colonies per well, when compared to the control, can be taken as a measure of the inhibitory capacity of the effector cells. Each effector cell/target cell combination was tested in at least 4 wells.

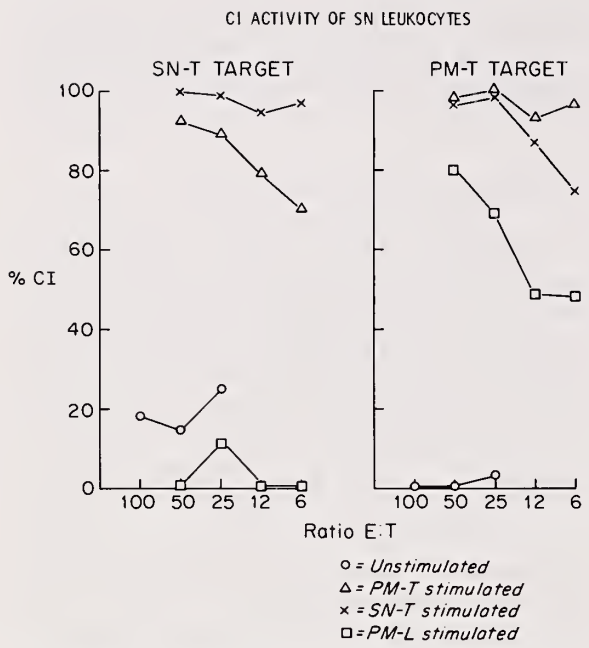
RESULTS

Stimulation of Leukocytes From BL patients by Autochthonous LCL Cells

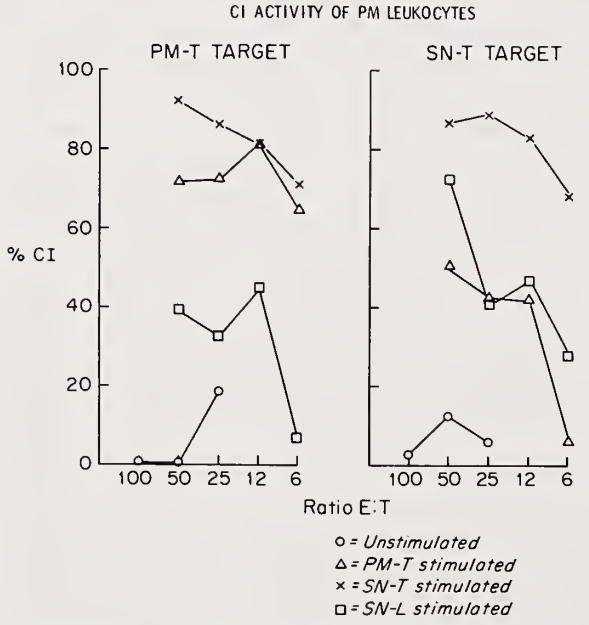
Positive stimulation in MLC tests was obtained in the 6 BL patients for which we had autochthonous LCL cells established in culture. The 2 patients described in this study were tested frequently in the autochthonous combination. Patient SN was tested for stimulation with SN-T cells on 7 occasions, and the response indices obtained ranged from 3.40 to 20.82, with an average for 7 experiments of 8.02 with a standard error of 2.57. In 4 experiments, the SN leukocytes were tested against PM-T stimulator cells and showed a very strong response, averaging 17.63 ± 4.88. Similarly, PM leukocytes showed a strong response to the autochthonous LCL cells and an even stronger response to allogeneic BL-derived lymphoblasts. In 9 experiments, PM leukocytes were stimulated by PM-T, with an average response index of 7.22 ± 2.01. Indices ranged from 2.07 to 20.48 in the autochthonous situation. In 5 tests of allogeneic stimulation with the SN-T cells, the response of PM leukocytes averaged 8.88 ± 2.54. Clearly, the degree of stimulation obtained by cocultivation of leukocytes from BL patients with autochthonous cultured lymphoblasts treated with mitomycin C was nearly equal to the degree of stimulation obtained in allogeneic combinations, indicating a very strong antigenic stimulation.

Effector Cell Activity After MLC

Text-figures 1 and 2 depict experiments in which the specificity of CI activity was tested before and after stimulation in MLC. In text-figure 1, leukocytes from patient SN were tested for CI activity against the autochthonous LCL target cell SN-T and an allogeneic target, PM-T, before stimulation in MLC. Without stimulation, no appreciable CI activity could be detected when compared to the CI activity of 2 normal control leukocyte preparations. After stimulation with the autochthonous SN-T LCL line, SN leukocytes displayed extremely strong CI activity against both the autochthonous and allogeneic BL-derived target cells. When the in vitro sensitization procedure was done with SN leukocytes and mitomycin C-treated PM-T cells, strong activity again was found against both target cells, though the greater activity was against the allogeneic line. However, when SN leukocytes were stimulated in MLC with PM-L cells (peripheral blood leukocytes of patient PM), the only appreciable CI activity was against the PM-T line. Thus, only activity attributable to histocompatibility antigens was detected. Similarly, in text-figure 2, PM leukocytes showed very slight CI activity against autochthonous or allogeneic target cells before stimulation. When PM leukocytes were sensitized in vitro with PM-T lymphoblasts, CI activity was considerable against both allogeneic and autochthonous LCL target cells. Some nonspecific activity was seen against the autochthonous line after stimulation of PM leukocytes in MLC with SN-L cells, but more activity was seen on the appropriate target cell (SN-T). In further experiments of this type, we have found only minimal CI activity against autochthonous target cells after MLC with allogeneic lymphocytes. These results indicate the presence of cross-reactive antigens on the PM-T and SN-T cells and the absence of such antigens on the peripheral blood leukocytes of these patients.



TEXT-FIGURE 1.—CI activity of unstimulated SN leukocytes calculated by comparison with number of colonies formed in presence of leukocytes from normal donors. CI activity of stimulated SN leukocytes derived by comparison with CI activity of control culture of SN leukocytes and mitomycin C-treated SN leukocytes.



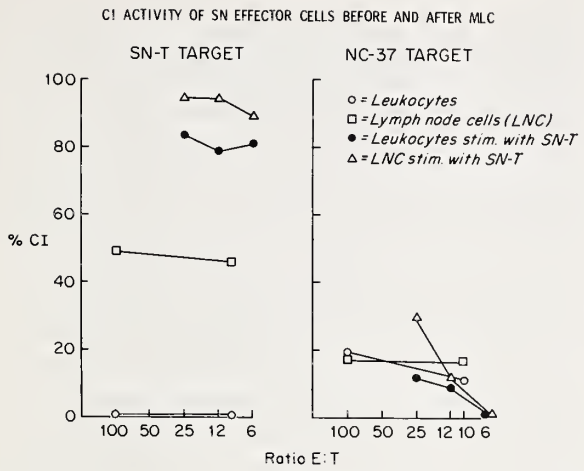
TEXT-FIGURE 2.—CI activity of unstimulated PM leukocytes calculated by comparison with number of colonies formed in presence of leukocytes from normal donors. CI activity of stimulated PM leukocytes derived by comparison with CI activity of control culture of PM leukocytes and mitomycin C-treated PM leukocytes.

Specificity of CI Activity Before and After Stimulation in MLC

A number of studies were carried out to determine the specificity of the CI activity seen post-MLC sensitization. One such experiment is shown in text-figure 3, where peripheral blood leukocytes, as well as lymph node cells from an inguinal node, of patient SN were tested for CI activity before and after in vitro sensitization. The peripheral blood leukocytes showed marginal activity against the autochthonous target cell line, while the lymph node lymphocytes showed significant reduction in colony formation of the SN-T target cells. In both cases, the degree of CI activity was assessed by comparison with the number of normal peripheral blood leukocytes. After stimulation in MLC with mitomycin C-treated SN-T cells, the SN effector cells had a marked increase in CI activity against the autochthonous target cell line, with the lymph node cells retaining the higher activity. Not shown are virtually identical results obtained with the same effector cells when tested on an allogeneic target cell, the PM-T line. In contrast, neither the peripheral blood leukocytes nor the lymph node cells, either before stimulation or after in vitro sensitization against the SN-T line, showed any significant activity against the NC-37 target cell line. Results were similarly negative with a second control target cell line, the PE-SS line, which, like NC-37, has no EBV antigens detectable by immunofluorescence. The lymph node cells, like peripheral blood leukocytes, showed increased incorporation of tritiated thymidine after stimulation in MLC with autochthonous SN-T cells. Virtually identical results, obtained by stimulation with LCL cells developed from subsequent tumors of patient SN, are not shown for reasons of clarity in text-figure 3. These lines showed in vitro stimulatory activity equivalent to the original SN-T line.

Failure of Autochthonous Fibroblasts to Stimulate Peripheral Blood Leukocytes

To determine if stimulation was due to the incorporation of a fetal calf serum antigen

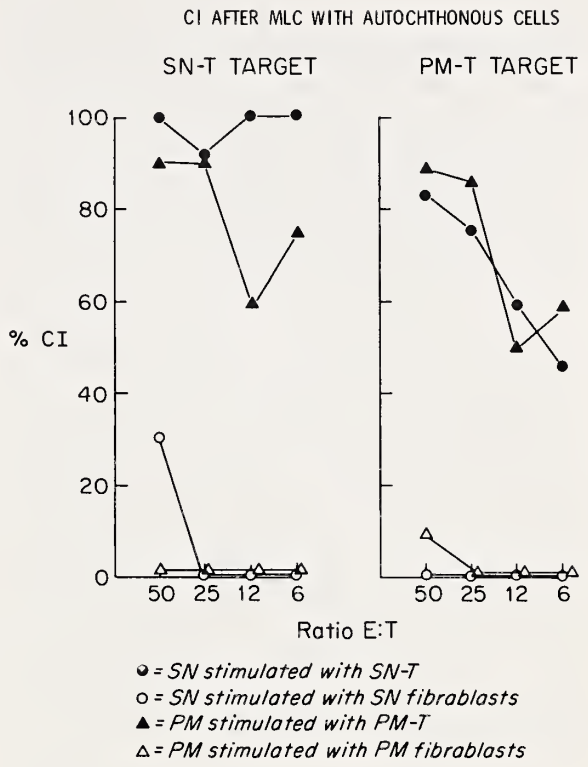


TEXT-FIGURE 3.—CI activity of SN leukocytes and LNC assessed by comparison with equivalent number of leukocytes from a normal donor. CI activity of stimulated effector cells derived by comparison with number of colonies formed in presence of control culture of SN leukocytes and mitomycin C-treated SN leukocytes.

into the membranes of the stimulator cells, or some other artifact of cultured cells, fibroblasts were also tested for stimulatory ability. Leukocytes from patients PM and SN showed no increase in DNA synthesis when cocultivated with autochthonous fibroblasts. Text-figure 4 shows the CI activity of these leukocytes. Stimulation with autochthonous LCL cells resulted in strong CI activity against the autochthonous and allogeneic target cells, while stimulation with autochthonous fibroblasts did not yield any appreciable CI activity. The slight CI activity at the higher effector:target ratios was probably due to a smothering effect of the large fibroblasts, as control experiments employing mitomycin C-treated fibroblasts alone showed only a slight and variable degree of CI activity. Apparently, the in vitro sensitization was not an artifact of using cultured cells.

DISCUSSION

These data indicate that LCL cells derived from BL tumors can stimulate autochthonous leukocytes in one-way MLC tests. An antigenic difference between the leukocytes and the cultured lymphoma cells clearly can be established from such experiments. Whether stimulation can be effected with non-cell-associated anti-



TEXT-FIGURE 4.—CI activity of stimulated effector leukocytes calculated by comparison with unstimulated control cultures consisting of leukocytes and mitomycin C-treated autochthonous leukocytes.

genic materials has not yet been established in the BL system. The experiments of Junge et al. (*21*) showed that remission-phase lymphocytes from infectious mononucleosis patients can be stimulated by autochthonous LCL cells, frozen acute phase mononucleosis cells, or cell-membrane fragments containing supernatants of the culture line. Since Clark et al. (*22*) have shown that no new species of macromolecule is sensitized by the lymphocytes during in vitro sensitization, any blastogenic factors in this system are more than likely cell-free fragments of the stimulator cell membrane.

One notable feature of the stimulation systems with LCL cells is the magnitude of the response of the lymphocytes. Han et al. (*11*) demonstrated that autochthonous stimulation with LCL cells resulted in rates of thymidine incorporation equivalent to those obtained by phytohemagglutinin stimulation. Likewise, our results indicate autochthonous stimulations

nearly equal to those obtained by one-way MLC with allogeneic LCL cells, and usually significantly greater than those obtained in straight mixed lymphocyte cultures (*20*). Since the responding lymphocytes display antigenic specificity in their effector cell activity, we must assume it represents a strong antigenic stimulation rather than some nonspecific mitogenesis. The presence of some cells, such as the samples of lymph node cells, that can mediate cytotoxic reactions against BL cells without prior stimulation, also indicates the presence of effector cells already presensitized. This is in accord with the findings of Powles et al. (*23*) stating that autostimulation in an MLC test with a leukemia cell can be enhanced by immunization of the patient with autochthonous tumor-cell vaccine. It is more difficult to explain the ability of LCL cells derived from normal donors to stimulate autochthonous lymphocytes (*10–13*) to such a high degree, but possibly normal individuals are sensitized, perhaps by EBV infection, to antigens which appear on LCL cells in culture.

The experiments on the CI activity of BL leukocytes after autochthonous stimulation enable us to study the nature of the antigenic systems involved in cellular reactions against BL cells in vitro. Leukocytes from BL patients stimulated with the autochthonous LCL cells had a very strong CI activity against the autochthonous target cell and also had strong CI activity against other target cell lines of BL origin. Activity was considerably lower against cell lines not having EBV antigens detectable by immunofluorescence, such as NC-37 and PE-SS. These findings suggest that the stimulatory antigens are in some way associated with the EBV-associated antigens.

Effector cells from in vitro sensitization were shown in a number of studies (*15–17*) to have specific cytotoxic activity. However, these systems are not without nonspecific aspects (*24–26*) that can yield cytotoxic activities in addition to the specific immunologic reactions. In our experiments and similar ones performed by Dr. Michael Steel (personal communication), there are certain nonspecific aspects in addition to the clearly specific cytotoxic activity seen after autochthonous stimulation. As previously reported (*18*), phytohemagglutinin-stimulated lymphocytes from normal donors as well as BL patients show strong activity in the CI test. Furthermore, BL leukocytes show some activity after autostimulation, although this activity is usually quite low, against non-EBV-producing cell lines, and occasionally some activity against autochthonous target cells after stimulation with allogeneic leukocytes. Interpretation of the CI activity against the so-called "EBV-negative" cell lines is clouded by the findings that such cell lines appear to contain EBV-specific DNA (*27*). Therefore, possibly what appears to be nonspecific activity in this sytem may be low levels of specific activity to EBV-associated antigens.

The antigens involved in stimulation clearly are not artifacts of cells from tissue culture as normal fibroblasts in culture fail to exert a stimulatory effect either in DNA synthesis or subsequent cytotoxic activity. Green and Sell (*10*) performed experiments to rule out the presence of fetal calf serum or other exogenous antigens in such stimulation systems. The failure of fibroblasts to sensitize autochthonous lymphocytes is in contrast to the report of Cohen et al. (*28*) who found that autosensitization on autochthonous fibroblasts could be accomplished. This discrepancy may be due to the technically different approaches used, the difference between the animal model systems and human systems, or possibly to the presence of C-type viral antigens in rodent fibroblasts. Most likely, the antigens involved in both the stimulation of autochthonous BL leukocytes and target cell sensitivity are either tumor-associated antigens related to the BL origins of these cell lines or antigens related to EBV, or both.

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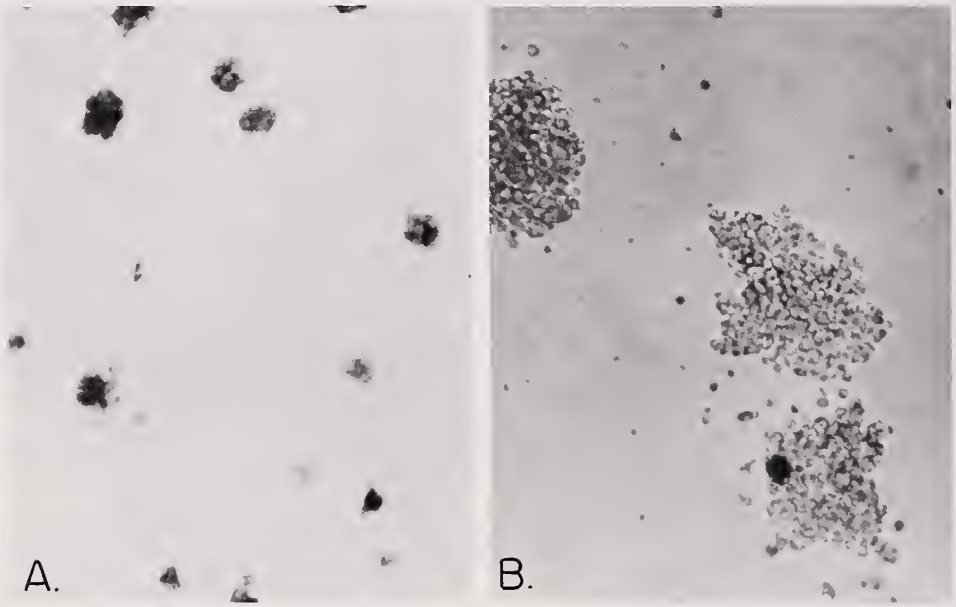

FIGURE 1.—LCL colonies used in CI test. Colonies of one of the LCL's (PE-SS) used in the CI assay. *A:* Colonies as they are seen for counting at low magnification (× 25). *B:* The close-packed nature of the colonies at higher magnification (× 40). Colonies were photographed 8 days after the seeding of a single-cell suspension.

Expression of Non-Tumor-Specific Antigen in Human Tumor Cells [1]

P. Koldovsky *and* J. Weinstein, *Wistar Institute and Division of Neurosurgery, Medical College of Pennsylvania, Philadelphia, Pennsylvania 19104*

SUMMARY—The expression of organ-specific antigens was studied in short-term cell lines in monolayer from brain tumor, brain, and skin of each patient. In addition, that of cancroembryonic antigen was studied in short-term cell lines from digestive tract tumors and skin of the same patient, as well as in cell lines derived from other embryonal organs.—Natl Cancer Inst Monogr 37: 33–35, 1973.

EXPERIMENTAL TUMORS induced by the same oncogenic virus exhibit cross-reacting tumor-specific transplantation antigens (*1*). In human tumors, antigens detected by cytotoxic reactions in vitro cross-react within the same clinical type of tumors (*2*). This cross-reactivity could be caused by: 1) the same virus being responsible for these animal tumors; or 2) the antigen detected in the human tumors differing in nature from animal tumors.

A tumor-associated antigen in humans can be defined as an antigen eliciting detectable reactions only against the tumor, leaving normal tissue unharmed. This definition includes various membrane-associated antigenic changes, such as 1) multiplication of normal antigen to the detectable level; 2) geographic rearrangement of antigens; and 3) close binding of extracellular proteins, budding viruses, bacteria, and mycoplasma bound to the cell membrane, organ-specific antigens, and embryo antigens.

ORGAN-SPECIFIC ANTIGENS

After malignant transformation, many, if not all, antigenic characteristics of the original cells are preserved so that organ- and/or tissue-specific antigens are still present. It is difficult to say which tissue does not have organ- and/or tissue-specific antigens. To illustrate this point, extensive studies on the surface antigens of the white blood cell population revealed the presence of antigens specific for B- and T-type cells (*3*).

To study organ-specific antigens, we selected the brain, an organ with well-known antigens and a relatively high incidence of tumors. We prepared short-term cell lines in monolayer from brain tumor, brain, and skin of each patient. We used the microcytotoxicity technique (*4*) and followed the cytotoxic activity of the peripheral white blood cells, the serum, and the blocking activity of the serum.

Cytotoxic activity of peripheral white blood cells against the tumor cells was found in only a few cases. When immune cells from one patient were cytotoxic against his tumor cells, these immune cells were found to be cytotoxic against tumor cells from all other patients. When antisera were prepared in guinea pigs by immunization with normal human brain tissue and made anti-brain specific by absorption (numerous human cell lines, white blood cells pooled from 30 healthy donors), such antisera detected cross-reacting antigen(s) in cells derived from normal brain and brain tumors. The fact that this antigen is organ specific was suggested by the finding that such serum also reacts with brain-derived cells from other species (table 1).

[1] Presented at Conference and Workshop on Cellular Immune Reactions to Human Tumor-Associated Antigens, held at the National Cancer Institute, June 8 and 9, 1972.

TABLE 1.—*Cytotoxic activity of guinea-pig anti-human brain serum absorbed with human leukocytes and various human cell lines*

Cells	End dilution	Cells	End dilution
B25	8	Sheep brain	32
	32	Mouse brain	16
N25	<2	Mouse kidney	<2
2RA	<2	S15	<2
BT13	<2	BT17	<2
B11	64	BT12	32
N11	<2	WI38	<2
B9	16		

The best serum for detection of a brain-specific antigen was one we obtained from a patient with a cerebroperitoneal shunt. This serum reacted specifically with cells derived from the brain and the brain tumor, and this again proved the presence of an antigen common to brain and brain tumors (table 2).

TABLE 2.—*Growth inhibition activity of patient serum #44 for human cells in vitro*

Cells	Endpoint activity	Cells	Endpoint activity
B8	8	B23	4
B9	8	BT23	4
B11	16	C23	<2
N11	<2	BT17	<2
B25	8	BT13	<2
BT25	16	BT12	8
N25	<2	WI38	<2

EMBRYO ANTIGENS

The other group of tumor-associated antigens which can be responsible for wide cross-reactivity are so-called cancroembryonic antigens. Such antigens can be divided into different groups on the basis of biologic properties. However, we are interested in the type of antigen responsible for cell membrane-associated reactions. We selected tumors of the digestive tract. Again, we prepared short-term cell lines from the tumors and skin of the same patient, as well as cells derived from the following embryonal organs: the lung, intestine, skin, kidney, and brain. We used several embryos, all of which were about 3 months.

We found that peripheral white blood cells are cytotoxic for the tumor cells from the same as well as from different patients. The sera contained cytotoxic antibodies in very low titer; however, the blocking activity of the same sera was higher. Furthermore, sera from some pregnant women were cytotoxic for these tumor cells in vitro (table 3). The same sera from

TABLE 3.—*Activity of human sera against adenocarcinoma cells in vitro*

Source of serum and serum No.	Adenocarcinoma cells	
	BT13	BT17
Adenocarcinoma patients:		
1	16	<2
2, 3, 12	8	NT*
11, 13	8	<2
14	4	NT
Pregnant women:		
4, 7	2	<2
5, 8	2	NT
6, 10	<2	NT
9	4	<2
Carcinoma patients:		
18, 25, 44	<2	

*NT = not tested.

tumor patients or from pregnant women that were cytotoxic against tumor cells were also cytotoxic against cells derived from the embryo intestine. Cells derived from the skin and kidney of the same embryo showed no reaction. Antisera prepared by immunization and proper absorption in guinea pigs had cross-reacting antigen between cells derived from the digestive tract tumor and embryo intestine cells (table 4).

TABLE 4.—*Activity of GP antisera against human embryo and tumor cells*

Cells	Antisera					
	Nonabsorbed		Absorbed with normal tissue		Absorbed with embryo or tumor tissue	
	Anti-BT13	Anti-EI	Anti-BT13	Anti-EI	Anti-BT13	Anti-EI
Embryo intestine	256	256	16	16	<2	<2
Embryo lung	256	512	<2	<2	<2	<2
BT13	512	256	32	16	<2	<2
C13	256	512	<2	<2	<2	<2
CA2	NT*	NT	16	8	NT	NT
CA4	NT	NT	16	8	NT	NT

*NT = not tested.

The antigens associated with the 2 tumors discussed above are interesting from several viewpoints. The brain-specific antigen in brain tumors represents a warning that, in such tumors, complications with encephalitis are possible, and that immunotherapy should not be recommended. The presence of embryo antigens in human digestive tract tumors is tempting as a possible immunotherapeutic approach.

Both antigens (brain and canceroembryonic) can be at least partially responsible for cross-reaction among some human tumors. However, this does not mean that these antigens are the only specific antigens in these tumors. The presence of a true tumor-specific antigen is likely. For example, in the brain tumors, though a reaction of white blood cells was present against tumor-derived cells, no reaction was detected against normal brain cells.

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Some Problems Involving In Vitro Cellular Cytotoxicity Assays [1, 2]

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SUMMARY—Quantitative in vitro studies of cellular cytotoxicity are hindered by technical problems and biologic variables. Objective and reliable counting of target cells will be much easier if the cells can be labeled with an isotope. The use of tritiated thymidine for this purpose results in significant killing of those cells that incorporated the largest amount of isotope. Much better cell survival has been obtained with tritiated uridine. Omission of fetal calf serum from the cultures at the time that lymphocytes are added, as carried out in some laboratories, appears to be extremely detrimental to the culture in that a significant number of target cells are released from the surface even after very short exposures to media that do not contain serum. Nonspecific lysis of target cells by normal lymphocytes is variable and should be considered when one uses this combination as a control for target cell killing by immune lymphoid cells.—Natl Cancer Inst Monogr 37: 37–39, 1973.

VARIOUS METHODS have been used to demonstrate in vitro cellular cytotoxicity: colony inhibition (*1*), chromium-51 release (*2*), and radioactive thymidine release (*3*). It is unnecessary to point out the need for a readily reproducible, quantitative in vitro assay for cellular cytotoxicity in the field of tumor immunology. Rather than review our published (*4*) and unpublished positive data, we would like to present various problems with the cellular cytotoxicity assay that we have found in our laboratory. The data to be presented pertain both to human neoplasms and murine tumor systems.

The major thrust of our effort has been to develop a microassay utilizing radioactively labeled target cells. The main advantages of methods involving the release of radioactive substances from target cells are easy quantitation and the measurement of cell death and lysis rather than inhibition of cellular growth and division. Tritiated thymidine is stably incorporated into nuclear DNA and would logically appear to be the compound of choice. However, in our experience, a major problem with tritiated thymidine has been radioactive cytotoxicity which is related to both the specific activity and the amount of incorporation of the label. This radioactive killing of target cells is most marked in murine tumor systems, ranging from 30% to 60%, but it also occurs to lesser degrees with human tumor cells. Despite extensive modifications of labeling procedures, we have been unable to eliminate this detrimental radioactive effect and yet obtain a satisfactory label with tritiated thymidine. Consequently, we searched for another technique and found that, if properly used, tritiated uridine satisfied our requirements (text-fig. 1). Pulse labeling with tritiated uridine for 3 hours resulted in 50% of the intracellular tritiated uridine being in the trichloroacetic acid (TCA)-precipitable pool. This percentage rapidly rose to 95% during the subsequent 24-hour period. However, during the 1st day, approximately 20% of the

[1] Presented at Conference and Workshop on Cellular Immune Reactions to Human Tumor-Associated Antigens, held at the National Cancer Institute, June 8 and 9, 1972.

[2] Conducted under Public Health Service contract NIH 69–2061 within the Special Virus Cancer Program of the National Cancer Institute.

[3] Department of Surgery.

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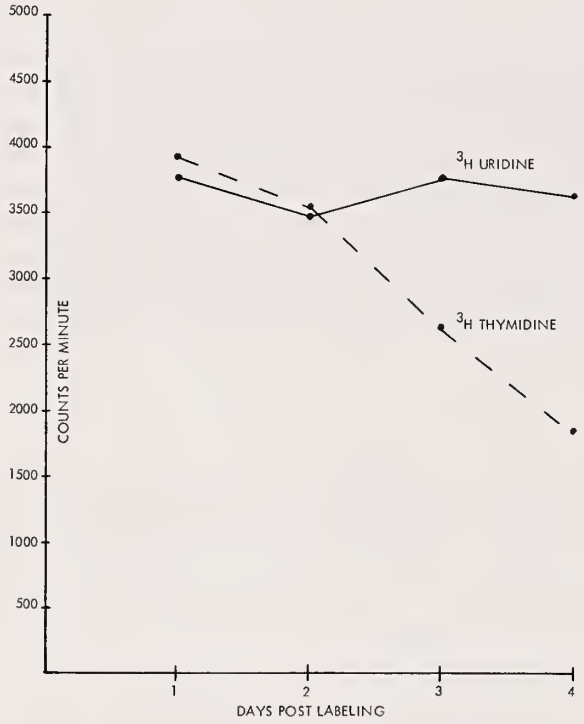
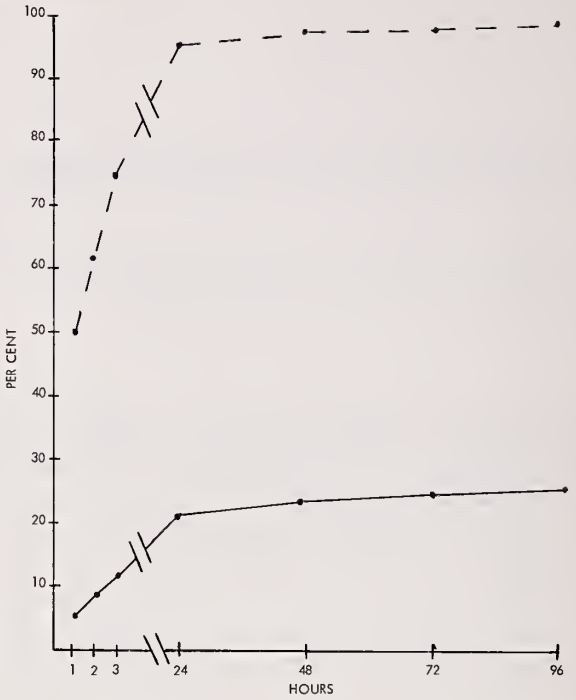
labeled intracellular uridine leaked from the cell. After this initial 24-hour period, the TCA-precipitable fraction of tritiated uridine remained stable for at least 4 days in culture (text-fig. 2). In addition, short pulse labeling of tritiated uridine-labeled tumor cells with carbon-14 thymidine demonstrated that these cells remain in log phase of growth and do not differ significantly from unlabeled cells.



TEXT-FIGURE 1.—Comparison of radioactive killing due to tritiated thymidine (specific activity 17 Ci/mM) versus tritiated uridine (specific activity 28 Ci/mM) on murine 3-methylcholanthrene-induced sarcoma cells.

One of the points made by the Hellströms (*1*) is the initial cocultivation of target cells and lymphocytes in the absence of fetal calf serum (FCS). Our data indicate that incubation of preplated cells alone in the absence of FCS for periods as short as 5 minutes resulted in substantial loss of target cells (text-fig. 3). However, 5% FCS did not interfere with cellular cytotoxicity and yet was sufficient to maintain the monolayer of target cells.

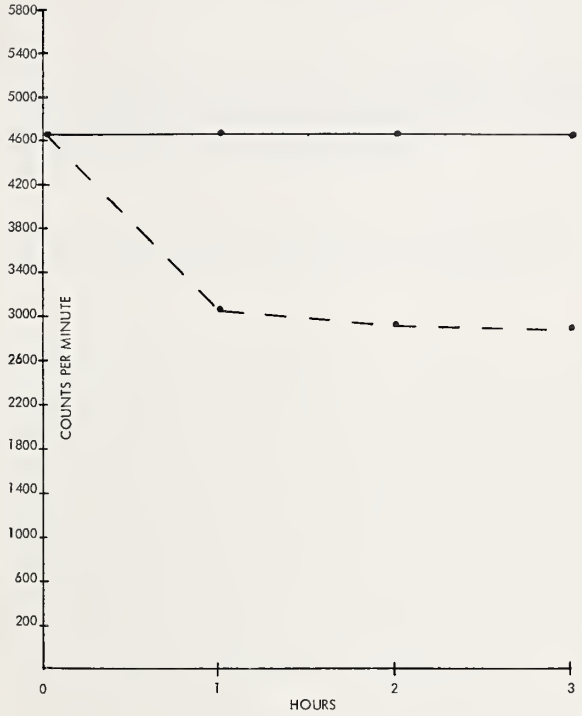
Another major problem with the reproducibility and quantitation of in vitro cellular cytotoxicity is the variability of the "nonspecific"



TEXT-FIGURE 2.—Stability of tritiated uridine label. Murine 3-methylcholanthrene-induced sarcoma cells were pulse-labeled for 3 hours with tritiated uridine and chased with cold media for 96 hours. ●——● percent of total intracellular uridine present at time zero subsequently released into supernatant. ●- - - - - - ● percent of intracellular uridine present in TCA-precipitable fraction.

lysis of target cells by normal lymphocytes. In our experience, this ranged from 0 to 100%, with the average being 20%, and increased with the duration of the assay. The interpretation of this phenomenon is at best difficult and, because of its rather frequent and unpredictable occurrence, cannot be dismissed as due to technical error in all or even most instances. In human systems, in particular, the need for multiple, well-chosen controls is evident. Another facet of this problem is the presence of so-called "nonspecific" blocking factors in sera from normal donors. Whether these serum factors are nonspecific or highly specific is only a matter of conjecture, since experimental evidence is lacking.

To eliminate, or at least reduce, nonspecific cytotoxicity, we have tried 2 approaches. One was to shorten the assay. For example, by terminating the assay at the end of 48 hours



TEXT-FIGURE 3.—Loss of target cells following culturing without FCS. Tritiated uridine-labeled murine 3-methylcholanthrene-induced sarcoma cells plated for 24 hours in 30% FCS, washed, and cultured in either 10% (●————●) or % (●--------●) FCS.

rather than at 96 hours, immune killing was increased from 19% to 45% and nonimmune killing was reduced from 44% to 7%. The other approach was the removal of adherent cells from the total lymphoid cell population. When adherent lymph-node cells were removed from the total lymph-node cell population, immune killing was increased from 9% to 47% and nonimmune killing was reduced from 34% to 14%. (Nonimmune killing was calculated with the use of target cells alone as the control; immune killing was calculated with the use of normal lymphocytes and target cells as the control.)

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Prelabeling Target Cells With ^{3}H-Proline as a Method for Studying Lymphocyte Cytotoxicity [1, 2]

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SUMMARY—Some difficulties we encountered in working with the in vitro assay for lymphocyte cytotoxicity described by Takasugi and Klein (and modified by the Hellströms) were 1) the time-consuming evaluation by visual counting, 2) the uncertainty of interpretation in terms of cell destruction versus inhibition of cell proliferation, 3) the selection of suitable target cells, and 4) the purification of human blood lymphocytes. Prelabeling of the target cells with ^{3}H-proline permitted rapid evaluation by liquid scintillation counting, and the results were interpreted as reflecting cell destruction rather than inhibition of cell proliferation. The isotope was adequately retained by viable cells during incubation, and isotope released from destroyed cells was not reutilized. Cells of the T-24 line, derived from a carcinoma of the bladder, have been shown by Bubeník et al. to be specifically destroyed by lymphocytes from patients with bladder cancer. With ^{3}H-proline-labeled target cells, our preliminary work tends to support their results, provided the lymphocytes are purified by the same method as they use, i.e., erythrocyte sedimentation with gelatin, osmotic shock with ammonium chloride, and filtration over a nylon column. By contrast, the cytotoxic effects of lymphocytes prepared by the more convenient Ficoll-Hypaque method were less specific; i.e., lymphocytes from bladder cancer patients were cytotoxic on control target cells as well as T-24.—Natl Cancer Inst Monogr 37: 41–48, 1973.

THE IN VITRO assay for lymphocyte cytotoxicity as originally described by Takasugi and Klein (*1*) and subsequently modified by Hellström et al. (*2*) is now used widely to measure cell-mediated immunity to cell-surface antigens. The test is based on the destruction or detachment of adherent target cells by lymphocytes that are sensitized to their surface antigens, and it is evaluated by counting the target cells that remain attached. Working with tests such as these that use adherent target cells, we, as well as others [*see* (*3*)], have encountered nonimmunologic factors which may give rise to misleading results. For instance, the number of target cells may be reduced by the "crowding" effect of large numbers of lymphocytes, by nonlymphocytic components of the lymphocyte[4] suspension (which vary depending on the method of preparation), and by mechanical or chemical effects during washing and staining. Conversely, the target cells may proliferate during incubation, and there may be a "feeding" effect of the lymphocyte suspension. Another disadvantage of the test as it is commonly practiced is the tedious visual counting of the remaining target cells. We present here results that illustrate some of the difficulties and a new method of labeling the target cells which permitted us to evaluate the test

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[3] We are indebted to Dr. Jørgen Fogh for his help in maintaining tissue-culture cell lines free of mycoplasma.

[4] The term "lymphocyte" is used here for convenience. Obviously, even the purest preparations contain nonlymphocytic cells such as granulocytes and macrophages.

by measuring remaining label rather than by counting the number of remaining target cells.

MATERIALS AND METHODS

Until recently the method we used was quite similar to that of the Hellströms (*2*). Monolayers of established or short-term tissue-culture cell lines were incubated with 0.25% trypsin [5] to produce a single cell suspension. The cells were washed in medium with fetal calf serum (FCS), suspended at a concentration of 10^4 viable cells/ml, and dispensed in samples of 10 μl with a Hamilton syringe [6] into the wells of Falcon Microtest II plates [7] containing 0.1 ml of complete minimal essential medium [8] (MEM) with 10% FCS.[9] The target cells were allowed to attach to the bottom of the wells by incubation overnight at 37° C in humidified air with 5% CO_2. The plates were then washed twice by inverting them, shaking out the medium, and refilling the wells. Lymphocytes were added at varying concentrations in 0.1 ml complete MEM with 10% FCS/well, and the plates were further incubated for 2 or 3 days. The plates were then washed twice to remove lymphocytes and nonadherent target cells. The remaining cells were fixed in methanol, stained with Giemsa, and counted. Usually, each lymphocyte concentration was tested in 8 replicates. At the time the lymphocyte suspension was added (0 time), the number of adherent target cells was determined.

[5] Bacto-trypsin, Difco Laboratories, Detroit, Mich. Enzyme rehydrated to 0.25% with Puck's saline A, Gibco, Grand Island, N.Y.

[6] Hamilton Microliter Syringe, used with Hamilton Repeating Dispensor, Hamilton Co., Whittier, Calif.

[7] Falcon Microtest II plate and Falcon Microtest II lid, Falcon Plastics Co., Catonsville, Md.

[8] Complete MEM was Gibco minimal essential medium (Eagle) with Earle's balanced salt solution (EBSS) containing L-glutamine at 2 mM (#503L), nonessential amino acid solution at 1% of 100× solution (#114), and penicillin-streptomycin at 200 U and 200 μg/ml, respectively (#514).

[9] FCS, Gibco. Complete MEM was supplemented with 15% noninactivated serum for maintaining the cell cultures and with 10% heat-inactivated serum (30 min at 56° C) for the cytotoxicity assays.

RESULTS AND DISCUSSION

The washing procedure is one factor that can reduce the number of target cells. Table 1 shows the effects of different washing conditions. Three types of solutions—EBSS, phosphate-buffered saline (PBS), and PBS with 5% FCS—were used. The fluid was removed either by gentle suction or by inversion and gentle shaking. The target cells were plated at a concentration of 200 cells/well and incubated overnight before the plates were washed. The addition of FCS to the washing fluid was tested because protein, in addition to divalent cations, facilitates adhesion of cells to surfaces (*4*, *5*). As expected, the addition of FCS increased the number of remaining target cells. There was no significant difference between the suction and inversion methods when FCS was present. When EBSS and PBS were used without FCS, approximately half the cells were lost, slightly more with suction than with inversion. In all subsequent experiments, the washing fluid was supplemented with FCS.

TABLE 1.—*Effect of washing technique on the remaining number of adherent target cells (HT 144, malignant melanoma, passage 58) in Microtest II plates*

Washing solution (20 hr after seeding 200 cells/well)	Number of target cells ±SEM* remaining after washing by:	
	Suction	Inversion
Earle's BSS	109±7[a†]	129±6[d]
PBS (Mg^{++} and Ca^{++} free)	122±6[b]	140±10[e]
PBS with 5% FCS	288±13[c]	294±13[f]

*Standard error of the mean of 8 replicate samples.

†Significance according to 2-tailed Student's *t* test; a=b; d=e; c=f a≠d $P<0.05$; b≠e $P<0.05$; b≠c $P<0.0001$; e≠f $P<0.0001$.

Nonspecific cytotoxicity of lymphocyte preparations was another factor causing loss of target cells. Initially we used a modification of the simple and efficient Ficoll-Hypaque (Isopaque) method described by Böyum (*6*) to purify lymphocytes. However, lymphocytes from normal donors prepared by this method were often cytotoxic for target cells. This is illustrated in table 2 where 2 methods of purifying lymphocytes are compared. Trypsinized cells of a culture derived from a pulmonary metastasis of an osteogenic sarcoma (passage 4) were used

as target cells (80 cells/well). The test was performed as outlined above. Lymphocytes were prepared from heparinized blood of a normal donor (L.D.) by either the Ficoll-Hypaque method or the plasmagel-nylon column method used by the Hellströms (*2*). When the lymphocytes were added, there were 72 target cells per well. After 48 hours of incubation in medium alone, the cell count had slightly increased. While the lymphocytes purified by the Ficoll-Hypaque method (>99% mononuclear cells) reduced the number of target cells by almost 50%, the lymphocytes prepared by the plasmagel-nylon column method (with 88% mononuclear cells) caused no loss of target cells. Further experiments showed that the use of defibrinated rather than heparinized blood reduced the "nonspecific" cytotoxicity of lymphocytes purified by the Ficoll-Hypaque method and that filtration through a nylon column almost completely eliminated this cytotoxicity. However, as we shall see, there are still some questions regarding the use of the Ficoll-Hypaque technique to prepare lymphocytes for the lymphocyte cytotoxicity test.

TABLE 2.—*Nonspecific cytotoxicity of lymphocytes purified by the Ficoll-Hypaque method*

Conditions of assay	Number of target cells remaining*
No lymphocytes	87.9±6.1†
Ficoll-Hypaque lymphocytes‡	47.0±5.0
Plasmagel-nylon column lymphocytes‡	87.0±5.2

*(B.M.) osteogenic sarcoma cells, 0 time was 71.2±2.9.
†Standard error of the mean on 8 samples.
‡200,000 lymphocytes from heparinized blood (L.D., normal), (F.H.—1% polymorphonuclear leukocytes, plasmagel—12% polymorphonuclear leukocytes) incubated 48 hours.

Often there was considerable proliferation of target cells during the incubation period, both with short-term cultures and long-established cell lines. An example is provided in table 3. In this experiment, the number of target cells (a line of fibroblasts derived from normal skin) doubled during the 48-hour incubation period. The increase was much smaller in the presence of autologous lymphocytes prepared from defibrinated blood by plasmagel sedimentation and nylon-column filtration. In other experiments, however, lymphocytes stimulated the proliferation of target cells.

TABLE 3.—*Effect of autologous lymphocytes on fibroblasts from normal skin*

Conditions of assay	Target cells remaining*
0 time	82.5±5.4†
48 hours—no lymphocytes	159.2±10.6
48 hours—lymphocytes‡	103.1±5.6

*Fibroblasts (M.C.) from normal skin.
†Standard error of the mean on 8 samples.
‡200,000 lymphocytes (M.C.).

In effect, the lymphocyte cytotoxicity assay as described by Takasugi and Klein (*1*) and modified by the Hellströms (*2*) measures both inhibition of proliferation and destruction of target cells. To obtain a more accurate estimate of target cell destruction, we investigated the use of labeled target cells. We were also motivated by the desire to eliminate the tedious procedure of visual counting.

When we considered which radioisotopes we might use for labeling the target cells, the following characteristics seemed desirable: 1) The isotope should be incorporated in amounts sufficient to effect adequate labeling of very small numbers of cells without prohibitive toxicity; 2) the isotope should be retained long enough to allow labeling of the target cells before plating so that only one, rather than many, samples had to be handled; and 3) the isotope, once released, should not be reutilized by target cells or lymphocytes.

We did not use ^{51}chromium because we believed it not ideally suited for prelabeling of monolayers prior to trypsinization and seeding into microplates. It was more suitable for measuring isotope release rather than retention [*see* (*3*)]. Experiments following the labeling method of Jagarlamoody et al. (*7*) showed that, with samples as small as ours, the amount of ^{3}H-thymidine that had to be incorporated was prohibitively high; a dose producing as little as 1 count per minute (cpm) per cell caused detachment of target cells. For the same reason, we decided against using ^{125}I-iododeoxyuridine. Cells attach poorly after they have been labeled with this isotope, and Cohen and Millar (*8*) found that labeling of already attached target cells resulted in only 1 cpm per cell. Labeled uridine did not seem attractive because of the rapid turnover of RNA and the problem of

reutilization during long incubation periods (*9*).

Although assays have been described that utilize the incorporation of labeled amino acids to measure the viability of target cells at the end of cytotoxicity tests (*3, 10*), little has been published on labeling target cells with amino acids before the actual test. Perlmann and Broberger (*11*) used ^{14}C-algal protein hydrolysate to prelabel human colon and fetal tissues. Perlmann and Holm (*3*) believe, however, that reutilization of labeled amino acids might be a considerable problem. This difficulty could be alleviated by addition of unlabeled amino acids to the medium used in the test. Eagle's MEM does not contain proline, a nonessential amino acid which is a constituent of proteins with relatively long half-lives. Therefore, we attempted to prelabel target cells by incubating them with ^{3}H-proline and then adding nonessential amino acids (including unlabeled proline) to the medium to prevent later reutilization of the already incorporated ^{3}H-proline when it is released by normal protein turnover or cell destruction. Small numbers of cells incorporated an adequate amount of label, the spontaneous release of the incorporated label was of a low order, and there was no reutilization of ^{3}H-proline. The procedure was as follows:

T-30 or T-60 tissue-culture flasks with monolayer cultures were incubated with 50 or 100 μCi/ml of ^{3}H-proline [10] in 5 or 10 ml of MEM with 15% FCS, glutamine, and penicillin-streptomycin, but without nonessential amino acids for 20–24 hours. The cultures were gently washed 3 times with 10 ml of warm medium to which 2% nonessential amino acids had been added. The cells were trypsinized (*see* above), and 100–1,000 labeled cells were distributed in portions of 10 μl into the wells of a Microtest II plate which contained 0.1 ml complete MEM with 10% heat-inactivated serum from human donors of blood type AB Rh+. The change from FCS to human serum was made because individual batches of commercially available FCS seemed to vary in quality and because we were concerned about possible blastogenic effects of FCS on human lymphocytes, problems that were also considered by Levy et al. (*12*) in their studies of lymphocytotoxic reactions to human brain tumors. After plating the target cells, we added lymphocytes in 0.1 ml of complete MEM with human AB Rh+ serum. The plates were then incubated for 60–64 hours. At the end of this period, we removed the medium by inverting the plates and shaking them lightly. They were then submerged in 37° C PBS with 5% FCS and emptied, and the process was repeated once more. After the plates had dried at 37° C (30–60 min), 0.2 ml Hyamine Hydroxide (Packard Instrument Co., Downers Grove, Ill.) was added to each well with a Cornwall (Cornwall pipettor with 1-cc syringe, Ace Scientific Supply Co., Linden, N.J.) automatic syringe equipped with Teflon valves and tubing. The plates were then incubated 15 minutes at 37° C to solubilize the remaining target cells. The contents of each well were then harvested by means of an apparatus which flushed the well with toluene PPO, dimethyl POPOP scintillation fluid (Packard Permablend I, Packard Instrument Co.), and the contents were transferred into scintillation vials. Since the scintillation fluid dissolved the plastic surface on contact, adequate recovery of the isotope in each well was ensured. The vials were allowed to cool for at least 30 minutes before counting to reduce chemo- and photo-luminesis. Currently, we are using 5–8 replicate samples at each lymphocyte concentration.

The retention of label at various times is shown in table 4. In this experiment we used the T-24 cell line established by Bubeník et al. (*13*) from a carcinoma of the bladder (and kindly supplied by Perlmann and O'Toole) and a line of fibroblasts derived from the normal skin of a melanoma patient (A.R.) in our laboratory. Monolayers of these 2 cultures were incubated for 24 hours with 100 μCi ^{3}H-proline (27 Ci/mM specific activity) per ml in a total volume of 5 ml in a Falcon 75-cm^2 tissue-culture flask. Replicate plates were seeded with 100 cells/well, and they were processed for both scintillation and visual counting at the indicated intervals. This isotope concentration

[10] Either New England Nuclear, L-proline (2,3-^{3}H) (N), specific activity 45.7 Ci/mM, New England Nuclear Corp., Boston, Mass., or Schwartz Bioresearch, L-proline (5-^{3}H), specific activity 27 Ci/mM, Schwartz Bioresearch Corp., Orangeburg, N.Y.

TABLE 4.—*Retention of label at various intervals after labeling target cells with ^{3}H-proline*

Incubation (hr)	T-24, bladder carcinoma		A.R., fibroblasts	
	Stained cells	^{3}H-cpm	Stained cells	^{3}H-cpm
0	(100)*	1225±89†	(100)*	408±25
20	99±13	942±105	68±3	310±16
44	85±8	895±109	72±4	248±11
68	80±4	485±74	72±1	160±9
92	59±9	321±29	70±4	151±7
116	47±6	99±12	63±2	130±3

*Calculated number of prelabeled target cells plated per well in Falcon Microtest II plate.
†Mean of 5–8 samples ± standard error of mean.

is the highest we have used so far. At this level, there was some loss of T-24 target cells, but not the fibroblast target cells, as time went on. The amount of label decreased gradually but remained adequate for scintillation counting. Even at 116 hours, there were still 2 cpm per target cell in a scintillation counting system where the efficiency was approximately 50% in a minimally quenched sample (A.E.S. of 0.77, Packard Tricarb). Subsequently, when working with the T-24 cell line, we have used 1,000 cells/well to obtain the density of target cells that is currently used by O'Toole et al. (*14*) in their assays with this cell line in the smaller (Falcon 3034) plates.

Table 5 shows the results of an experiment designed to show whether there is reutilization of ^{3}H-proline. In our assay (*see* above), the ratio of nonlabeled to labeled proline after extensive washing of the monolayer and then incubation of the target cells in a test medium containing unlabeled proline was $>10^6$:1. Therefore, we did not expect reutilization. T-24 cells were labeled for 24 hours with 100 μCi of ^{3}H-proline/ml, trypsinized, and adjusted to a concentration of 10^5 cells/ml. The suspension was divided into 2 parts, one which was used as such and the other which was frozen and thawed 4 times so that all remaining cells were permeable to trypan blue. Both parts were distributed into the wells in portions of 10 μl (containing 1,000 labeled viable cells in the untreated suspension and the same amount of isotope in the frozen-and-thawed suspension). At 24 hours after plating, the plates were washed. The wells that contained untreated cells gave 7048 cpm, and the wells that contained the cells remaining after freezing and thawing gave 232 cpm, which indicated the most cells had been destroyed and that label had been available for reutilization. No incorporation of label was observed, however, in wells where the frozen-and-thawed cell suspension had been mixed with 1,000 nonlabeled viable cells. The nonlabeled target cells did not prefer to utilize ^{3}H-proline released by dead target cells in a medium that contained much greater amounts of nonlabeled proline. This was also true for lymphocytes (table 5). The frozen-and-thawed suspension of labeled target cells was incubated in test tubes with and without lymphocytes, and the cell pellets were separated by centrifugation. No incorporation of label by lymphocytes was found.

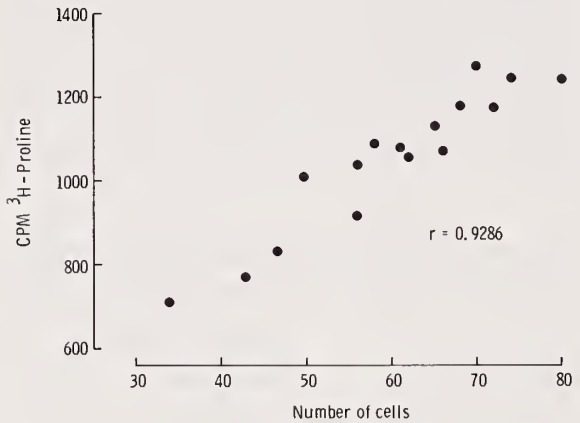
TABLE 5.—*Lack of reutilization of ^{3}H-proline released from labeled target cells destroyed by freezing and thawing in medium containing nonlabeled proline*

Condition of assay	^{3}H-cpm recovered†	
	Microtest plate	Test tube
10^3 labeled target cells*	7048±205	—
10^3 labeled target cells, frozen-thawed	232±10	656±40
10^3 labeled target cells, frozen-thawed $+10^3$ unlabeled target cells	193±9	—
10^3 labeled target cells, frozen-thawed $+500\times10^3$ lymphocytes	—	356±24

*T-24 bladder carcinoma labeled for 24 hours with 100 μCi/ml ^{3}H-proline before use.
†Average ± standard error of the mean.

The correlation between visual cell counts and the tritium counts is shown in text-figure 1. In this experiment, T-24 cells were labeled for 24 hours before plating. The plate was incubated for 64 hours and stained. After being visually counted, the individual wells were sep-

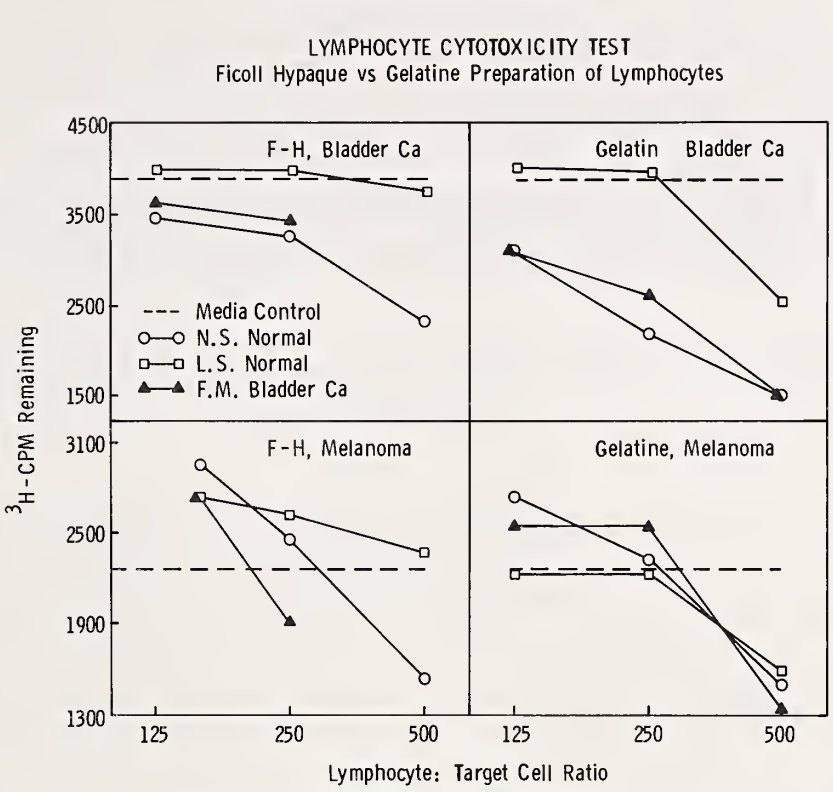
arated from one another and processed for scintillation counting. Each point in the diagram represents the mean of 8 replicates. There was a high degree of correlation between visual cell counts and cpm. Similar results were obtained with 2 other cell lines, an established line derived from a melanoma (HT-144) and a short-term culture of fibroblasts from normal skin (M.B.), the degree of correlation being 0.93 and 0.86, respectively. Considering that the lymphocytotoxicity assay, as evaluated by visual counting, measures both destruction of cells and inhibition of proliferation (*3*), one cannot expect a perfect correlation between the number of residual cells and the isotope counts where there is an appreciable degree of cell proliferation. However, it has been our experience that the ^{3}H-proline labeling reduces the proliferation rate.



TEXT-FIGURE 1.—T-24 (bladder carcinoma) tissue-culture monolayer cells were prelabeled with 100 μCi/ml ^{3}H-proline (specific activity 45.7 Ci/mM) for 24 hours before plating into a Microtest II plate. There were 16 test groups of 8 wells each, with different lymphocyte donors and sera comprising each. The plate was incubated 64 hours, then stained, and counted. After being visually counted, the individual wells were separated with a hot scalpel blade, and each well was processed for scintillation counting by solubilization of the well with Hyamine Hydroxide and toluene, PPO, dimethyl POPOP. Each *point* represents the average of 8 samples.

The isotope assay was used further in studies of lymphocyte cytotoxicity for T-24 cells, a line which was shown by Bubeník et al. (*13*) to be specifically destroyed by lymphocytes from bladder cancer patients. O'Toole et al. more recently have extended the study of this cell line (*14*).

Our preliminary experiments showed that lymphocytes from patients with bladder cancer were in fact cytotoxic for this cell line, whereas lymphocytes from most normal donors and donors with other types of cancer were not. However, the bladder cancer patients' lymphocytes appeared to be also cytotoxic for other target cells, not derived from carcinoma of the bladder. Because this was not consistent with the findings reported by Bubeník et al. (*13*) or O'Toole et al. (*14*), we investigated whether the Ficoll-Hypaque method of lymphocyte purification as we used it was responsible (text-fig. 2). Samples of defibrinated blood were divided into 2 batches and processed either with our modification of Böyum's method (*6*) (Ficoll-Hypaque gradient centrifugation followed by incubation on a nylon column for 30 min) or by the method currently used by O'Toole et al. (sedimentation of defibrinated blood by gelatin, hypotonic lysis of the red cells with Tris-ammonium chloride, and incubation on a nylon column for 30 min (*14*). Lymphocytes were obtained from a normal donor (N.S.) whose lymphocytes had shown nonspecific cytotoxicity for several target cell lines when prepared with our Ficoll-Hypaque method, from another normal donor (L.S.) whose lymphocytes had never shown cytotoxicity, and from a bladder cancer patient (stage T-2, superficial localized invasion), of the type whose lymphocytes were most likely to exhibit specific cytotoxicity (*14*). The lymphocytes from the bladder carcinoma patient (F.M.) and one normal donor (N.S.) showed cytotoxicity for both the bladder cancer cells and melanoma target cells when prepared by the Ficoll-Hypaque method. On the other hand, the gelatin-purified lymphocytes gave a clearer pattern of cytotoxicity for T-24 target cells, affecting the melanoma cells only at highest concentrations. This pattern was seen in 3 consecutive experiments with different target cells as controls. Therefore, we are currently using the method described by O'Toole et al. to purify human lymphocytes for the cytotoxicity assays. We believe that caution is indicated in interpreting the results obtained with lymphocytes purified by the Ficoll-Hypaque



TEXT-FIGURE 2.—T-24 (bladder carcinoma) and A.R. (melanoma) tissue cultures were prelabeled with 50 μCi/ml ^{3}H-proline for 24 hours before plating (1,000 cells per well in Microtest II plates). The Ficoll-Hypaque (F-H) or gelatin method was used to prepare lymphocytes from defibrinated samples of the same blood. Lymphocytes (99% mononuclear) were added at ratios of 500:1, 250:1, and 125:1 target cell. The plates were then incubated 64 hours before being processed for scintillation counting.

method, though we have not seen this cross-reaction when we used lymphocytes from patients with breast cancer or melanoma.

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Evaluation of a Cell-Mediated Cytotoxicity Assay Utilizing ^{125} Iododeoxyuridine-Labeled Tissue-Culture Target Cells [1, 2]

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SUMMARY—An in vitro lymphocyte cytotoxicity tissue-culture assay utilizing ^{125}iododeoxyuridine-labeled target cells is described. In a Gross virus-induced lymphoma of W/Fu rats, the peripheral blood lymphocytes of immunized animals lysed the ERTh/V-G cell line carrying the tumor-associated antigen. Release of ^{125}I into the medium was a direct reflection of cell death and lysis. Several difficulties have arisen with attempts to utilize this assay to detect cell-mediated tumor immunity in humans. Wide variation in the ability of lymphocytes from normal individuals to lyse these tissue-culture lines has been evident. Some, but not all, of this variability may be related to the method of lymphocyte purification. Before results can be interpreted from this and other assays for cell-mediated immunity, proper controls must be found.—Natl Cancer Inst Monogr 37: 49–58, 1973.

SINCE ROSENAU and Moon (*1*) first demonstrated that cell-mediated immunity can be measured by in vitro techniques, a variety of assays have been developed to measure this phenomenon (*2–10*). We previously reported on a modification of an in vitro assay utilizing ^{125}iododeoxyuridine (^{125}IUDR)-labeled tissue-culture target cells (*11*). Here we report some additional data in human tumor systems and discuss some of the technical problems inherent in this and possibly in all of these assays of cell-mediated tumor immunity.

^{125}IUDR is incorporated into target cell nuclear DNA in place of the sterically similar thymidine. An inhibitor of thymidylate synthetase, fluorodeoxyuridine (FUDR), is added so that cells preferentially take up the ^{125}IUDR in place of thymidine. This assay offers several potential advantages over many of the existing assays: The objectivity and ease of an isotopic method are preferable to the subjective visual counting methods. Using a gamma-emitting isotope as opposed to a beta emitter eliminates sample preparation for counting. This assay can be used both for monolayer and suspension cell systems as long as the cells proliferate and thereby incorporate ^{125}IUDR. After incorporation of this isotope into the target cell, there is no spontaneous release and very little reutilization (*12–14*). Therefore, release of this isotope into the supernatant is a direct reflection of cell death and lysis (*5, 10*).

Because of the work of Hirata (*15*) and later of Klein and Perlmann (*16*) indicating that trypsin can preferentially lyse target cells that have undergone immune membrane damage, we believed that trypsin might increase the sensitivity of this assay (*17*).

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[2] Supported in part by Public Health Service contract NIH-NCI 72-3227 from the National Cancer Institute.

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MATERIALS AND METHODS

Target cells.—To demonstrate the validity of the assay, immune response to inoculation of the Gross virus-induced lymphoma, (C58NT)D, into syngeneic W/Fu rats was studied (*18, 19*). The ERTh/V-G tissue-culture cell line, derived from embryonic thymus cells transformed by Gross virus, was used as the target cell line.

In the human studies, several established tissue-culture cell lines were used. Table 1 lists the origins and characteristics of these lines.

TABLE 1.—*Human tissue-culture cell lines used as target cells*

Cell line	Origin of cells	References	Characteristics
F-265	Normal lymphocytes	(*20*)	Lymphoblastoid
BT-20	Breast carcinoma	(*21*)	Epithelial
HT-39	Breast carcinoma	(*22*)	Epithelial
G-11	Breast carcinoma	(*22*)	Epithelial
LeCa	Malignant melanoma	(*23*)	Epithelial; produces melanin
Shailer	Malignant melanoma	(*24*)	Epithelial; produces melanin
2563	Lung adenocarcinoma	(*25*)	Epithelial
373	Colon carcinoma	(*26*)	Epithelial; produces carcinoembryonic antigen (*27*)
ET_s	Ewing's sarcoma	(*28*)	Lymphoblastoid, in suspension
9315	Spindle cell sarcoma	(*29*)	Fibroblastic
9320	Normal skeletal muscle	(*29*)	Fibroblastic

Attacker cells.—For the animal studies, 8- to 10-week-old W/Fu rats were immunized by subcutaneous inoculation of 1×10^8 (C58NT)D tumor cells as previously described (*18, 19*). Heparinized blood was obtained by cardiac puncture from rats 10–12 days after inoculation and from nonimmunized age-matched controls.

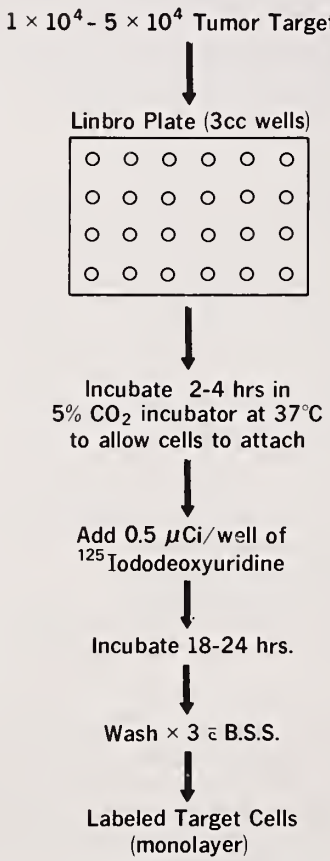
Heparinized peripheral blood was obtained from normal controls and from patients with neoplastic diseases who were not receiving chemotherapy or radiotherapy.

All peripheral blood lymphocytes were purified by the Ficoll-Hypaque gradient technique (*30*).

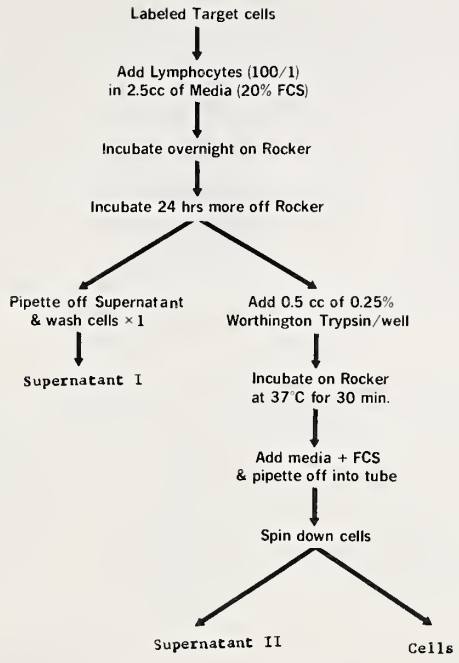
Assay technique (*11*).—Text-figure 1 outlines our method of prelabeling the target cells. The remainder of the assay was performed according to text-figure 2.

Since ^{125}IUDR is released only with cell lysis and is not reutilized by the target or attacker cell population (unpublished data), the amount of isotope released is a direct measure of cytotoxicity. Loss of adherence of intact labeled cells contributed little to the isotope found in supernatant I. Results were expressed as percent cytotoxicity and were calculated as follows:

$$\% \text{ cytotoxicity} = 100 - \frac{\text{cpm (counts per minute) in cells}}{\text{cpm in supernatant I} + \text{cpm in supernatant II} + \text{cpm in cells}} \times 100.$$


1 × 10⁴ - 5 × 10⁴ Tumor Target Cells
Linbro Plate (3cc wells)
Incubate 2-4 hrs in 5% CO₂ incubator at 37°C to allow cells to attach
Add 0.5 μCi/well of ¹²⁵Iododeoxyuridine
Incubate 18-24 hrs.
Wash × 3 ε B.S.S.
Labeled Target Cells (monolayer)

TEXT-FIGURE 1.—Labeling and washing technique to obtain target cells prelabeled with ^{125}IUDR. B.S.S. = balanced salt solution.


Labeled Target cells
Add Lymphocytes (100/1) in 2.5cc of Media (20% FCS)
Incubate overnight on Rocker
Incubate 24 hrs more off Rocker
Pipette off Supernatant & wash cells ×1
Add 0.5 cc of 0.25% Worthington Trypsin/well
Supernatant I
Incubate on Rocker at 37°C for 30 min.
Add media + FCS & pipette off into tube
Spin down cells
Supernatant II
Cells

TEXT-FIGURE 2.—Method of incubation of cells and final separation of supernatants from the cells. FCS = fetal calf serum.

The percent lysis by trypsin was calculated similarly:

$$\% \text{ cytotoxicity by trypsin} = \frac{\text{cpm in supernatant II}}{\text{cpm in supernatant II} + \text{cpm in cells}} \times 100.$$

All tests were performed in quadruplicate, and the means and standard errors (SE) calculated. This method of calculating cytotoxicity differs greatly from that used by most investigators with similar in vitro assays (*2, 3, 5, 10*). Most investigators calculate a cytotoxic index rather than true percent lysis, comparing the number of cells (or cpm) remaining in the experimental wells at the end of the assay with that in control wells. As will be illustrated in the discussion period to follow, this method can introduce some difficulties when low cell numbers remain in the wells. Therefore, we believed the percent cytotoxicity should be calculated from the amount of isotope actually released during the assay. The true level of lysis by immune and nonimmune lymphocytes could then be more accurately compared.

RESULTS

Labeling kinetics of the target cells were first examined in the W/Fu rat system. The cell number was an important variable (table 2). As the number of plated cells increased, the incorporation of ^{125}IUDR increased but, more importantly, the amount of cell death in the wells decreased. This was probably just a reflection of the cell numbers needed for optimal tissue-culture conditions. For most adherent cell lines, this has been a subconfluent monolayer.

TABLE 2.—*Effect of ERTh/V-G cell number on isotope incorporation*

Number of cells	Amount of isotope added per well (μCi)	cpm in cells		Percent of isotope released‡
		Initially*	At 48 hours†	
1×10^3	0.75	449±44	202±25	55
5×10^3	.75	2,045±23	1,133±24	40
1×10^4	.75	4,276±346	2,542±104	41
2.5×10^4	.75	11,087±365	8,296±253	27
5×10^4	.75	22,854±1,165	18,319±917	20

*cpm in cells after incubation with ^{125}I-IUDR for 20–24 hours and after 3 washes.
†Incubated at 37° C in a 5% CO_2 atmosphere with media only.
‡Same as spontaneous cell death.

Another variable was the ^{125}IUDR concentration. At high concentrations, ^{125}IUDR may produce radiation and chemical toxicity. For this assay, concentrations between 0.1 μCi up to 5.0 μCi per well gave no evidence of increasing toxicity (table 3). A third factor affecting the level of isotope incorporation was the presence of FUDR. Incorporation was 4 times higher in the ERTh/V-G cell line and from 2 to 10 times higher in the human cell lines in the presence of FUDR. There was no evidence of toxicity from the FUDR at 10^{-6}M (table 4).

TABLE 3.—*Effect of isotope concentration on the level of label incorporation into ERTh/V-G cells*

Number of cells	Amount of isotope added per well (μCi)	cpm in cells		Percent isotope released‡
		Initially*	At 48 hours†	
5×10^4	0.1	1,780±87	1,257±65	30
5×10^4	.25	4,097±139	2,366±178	41
5×10^4	.50	8,455±169	5,280±106	38
5×10^4	1.0	15,669±181	10,146±154	36
5×10^4	5.0	44,460±1,785	28,232±1,256	36
5×10^4	10.0	144,738±6,662	57,128±2,910	51

*cpm in cells after incubation with ^{125}I-IUDR for 20–24 hours and after 3 washes.
†Incubated at 37° C in a 5% CO_2 atmosphere with media only.
‡Same as spontaneous cell death.

TABLE 4.—*Effect of FUDR on the incorporation of ^{125}IUDR by ERTh/V-G cells*

Number of cells	Amount of isotope added per well (μCi)	Addition of FUDR (10^{-6}M)	cpm in cells		Percent spontaneous loss
			Initially	At 48 hours	
5×10^4	1	Yes	28,153±1,577	20,423±1,240	28
5×10^4	1	No	6,765±112	4,801±139	30

With the Gross virus-induced lymphoma in syngeneic rats, significant cytotoxicity was seen with the lymphocytes of immunized animals as compared to those of controls (table 5). Also, trypsin released a large amount of isotope from damaged cells as compared to controls. We believe this results from cell lysis by trypsin acting on immunologically damaged cell membranes. The cpm within each group were quite close with small standard errors, and the results in these assays were very similar from day to day. Sufficient isotope was incorporated into the ERTh/V-G cells so that samples had to be counted for only 1 minute.

TABLE 5.—*Cytotoxicity of peripheral blood lymphocytes of W/Fu rat against ERTh/V-G cells*

Attacking cells*	cpm			Percent cytotoxicity†			
	Initial supernatant	Trypsin supernatant	Cells	Trypsin‡		Total	
Immune	2563	132	100	56	59±2‡	97	96±1§
	2528	220	129	63		96	
	2488	200	150	57		95	
	2843	230	157	59		95	
	2628±79†	195±22	134±13				
Normal	2272	163	938	18	15±1	72	71±1
	2448	146	1010	13		72	
	1776	138	862	14		69	
	1758	136	729	16		72	
	2064±175	146±6	885±60				
Media control	947	211	1483	12	14±1	44	47±1
	961	259	1314	16		48	
	1287	215	1541	12		49	
	1086	264	1525	15		47	
	1079±79	237±14	1466±52				

*5×10^6 lymphocytes added to 5×10^4 target cells, giving attacker to target cell (A/T) ratio of 100:1.
†Results expressed as mean ± SE.
‡Percent of isotope in unlysed cells at end of assay released by trypsin.
§Significantly different from nonimmune controls, $P<0.001$.

The degree of cytotoxicity was related to the A/T ratio, with ratios of $\geq$25/1 giving significant cytotoxicity (table 6). In the human assays, ratios of 50/1 and 100/1 were used. At a given ratio, the incubation time was another variable influencing results. Significant activity was seen at 24 hours when high A/T ratios were used, but 48 hours was needed for lower ratios.

TABLE 6.—*Effect of the A/T ratio on immune cytolysis of ERTh/V-G cells*

Attacking cells	A/T ratio*	Percent lysis by trypsin	Total lysis (%)
Immune	10/1	10	25
	25/1	15	40†
	50/1	20	58
	100/1	25	75
Normal	10/1	10	30
	25/1	8	27
	50/1	10	29
	100/1	11	55
None (media control)	—	9	33

*5×10^4 target cells were added to each well; incubation was for 48 hours.

†At $\geq$25/1, immune cytolysis was significantly highter than that in nonimmune control, $P<0.001$.

The human tumor studies presented greater problems. Spontaneous cell death varied considerably among the cell lines (table 7), and this had to be determined before a particular cell line was used in this assay. Preliminary results indicated good specificity. Table 8 shows an experiment in which 2 patients with breast cancer had significantly greater cytotoxicity than a normal control or melanoma patient against BT-20, a line derived from breast carcinoma.

However, the melanoma patient did not show increased reactivity against the 2 melanoma cell lines.

TABLE 7.—*Spontaneous cell death in human cell lines*

Cell line*	Percent of isotope released over 48-hour incubation
9315	20 ± 4
9320	15 ± 4
LeCa	24 ± 2
BT-20	45 ± 2
ET_s	30 ± 5
F-265	55 ± 3

*5×10^4 cells labeled with 0.5–1.0 μCi ^{125}I-IUDR in each test.

As a further control in this and most subsequent experiments, lymphocytes were tested against F-265, a lymphoblastoid cell line derived from the lymphocytes of a normal donor. In the ^{51}Cr-release cytotoxicity assay, it had been previously found that the lymphocytes of most normal individuals cause lysis of this cell line and of other lymphoid cell lines (*31*). The data thus far indicate that the level of reactivity against F-265 may reflect the cytotoxic potential of an individual's lymphocytes. Patients with an immunodeficiency state or advanced cancer and those on immunosuppressive therapy have depressed reactivity. Although an insufficient number of tests have been analyzed to know if this correlation occurs also with the ^{125}IUDR assay, it was of interest that the 2 patients with high reactivity against BT-20 also had strong cytotoxic effects on F-265.

Table 9 shows an experiment with a larger number of target cells. The patient with breast cancer had significantly higher activity against 2 breast cancer lines, HT-39 and G-11, when compared to that in the normal control or to that in the other cancer patients. One patient

TABLE 8.—*Lymphocyte reactivity against BT-20 and other cell lines*

Lymphocytes			Target cells*			
Patient	Tumor type	A/T ratio	F-265	Shailer	LeCa	BT-20
M.O.	Melanoma	50/1	15/58	4/60	9/67	6/57
G.O.	Breast	50/1	43/80	—	—	*28/84*
M.K.	Breast	50/1	32/75	—	—	*23/83*
R.T.	Normal control	50/1	25/68	13/73	11/65	11/67
Media	—	—	9/40	12/50	8/43	9/45

*Results expressed as % lysis by trypsin/% lysis total.

TABLE 9.—*Lymphocyte reactivity against several cell lines*

Lymphocytes			Target cells				
Patient	Tumor type	A/T ratio	F-265	BT-20	HT-39	G-11	2563
L.W.	Lung	100/1	23/57	8/53	8/74	14/72	*68/88*
O.A.	Lung	100/1	29/62	10/75	9/70	17/68	25/64
M.K.	Breast	100/1	32/64	14/64	*18/84*	*24/81*	15/76
E.P.	Normal control	100/1	22/54	10/55	10/75	14/76	6/55
Media	—	—	11/39	14/43	7/41	9/57	5/28

with lung cancer had relatively high activity against the lung cancer line but not against the breast cancer lines.

As more tests were performed, many unexpected reactions were seen. Some cancer patients had strong reactions against cell lines derived from other types of cancer, and the lymphocytes of some normal individuals were highly cytotoxic (tables 10–12). Even the basic question of how to define a positive reaction became uncertain. In some tests, all the lymphocytes tested were significantly higher than the media control (e.g., tests against 2563 cells in table 12). It was also clearly unsatisfactory to use the results obtained with the normal lymphocytes as the "negative" control, since on some occasions these were as high or significantly higher (table 12) than those obtained with the cancer patients' cells. Because of these observations, the reactivity of several normal controls was examined. Lymphocytes from the same normal donor gave similar results on 3 separate occasions (table 13). Since this normal control and the others initially used in these assays were young (age 25–35 yr) laboratory personnel, we examined a group of nonlaboratory workers who were more closely age matched to the cancer patients (table 14). Clearly, the average reactivity, against the majority of the cell lines, of the younger control group was higher than that of the age-matched controls. Whether this difference was due to age, occupation, or other factors remains to be determined.

TABLE 10.—*Reaction of lymphocytes from patients with breast and colon carcinoma and from normal control against tissue-culture cell lines*

Lymphocytes			Target cells			
Patient	Tumor type	A/T ratio	F-265	HT-39	G-11	2563
M.L.	Breast	100/1	32/70	17/81	14/76	20/69
A.O.	Colon	100/1	13/52	5/57	4/50	2/40
H.H.	Colon	100/1	18/55	12/72	11/64	6/48
R.S.	Normal control	100/1	23/61	14/80	13/72	13/61
Media	—	—	11/49	13/66	6/45	4/36

TABLE 11.—*Reactivity of lymphocytes of a breast carcinoma patient against the lung adenocarcinoma cell line*

Lymphocytes			Target cells				
Patient	Tumor type	A/T ratio	F-265	BT-20	HT-39	G-11	2563
M.J.	Breast	100/1	37/76	7/53	12/75	17/72	10/69
M.K.	Breast	100/1	29/68	8/56	16/83	24/80	*17/82*
W.A.	Colon	100/1	19/57	6/41	11/69	13/65	5/62
L.F.	Normal control	100/1	27/67	6/51	20/82	20/75	6/66
Media	—	100/1	12/45	10/48	13/63	27/65	5/55

TABLE 12.—*Reactivity of lymphocytes from normal donor exceeding that of cancer patients against tissue culture lines*

Lymphocytes			Target cells	
Patient	Tumor type	A/T ratio	2563	373
F.O.	Lung	100/1	7/43	5/44
A.J.	Colon	100/1	4/36	5/41
T.C.	Normal control	100/1	*13/52*	*7/51*
F-265	—	100/1	7/36	5/32
Media	—	—	7/29	13/41

TABLE 13.—*Reproducibility of reactivity of the lymphocytes of a normal donor, W.B., against several cell lines*

Experiment	Target cells*					
	F-265	BT-20	HT-39	G-11	2563	373
1	12	33	31	50	51	12
2	10	33	29	44	37	2
3	13	33	22	47	55	7
Mean + SE	12±1	33±0	27±3	47±2	47±5	7±3

*A/T ratio 100:1; results expressed as total % lysis above media control.

TABLE 14.—*Summary of reactivity of normal controls and of cancer patients against several human cell lines*

Attacker cells*	Target cells†					
	F-265	BT-20	HT-39	G-11	2563	373
Normal laboratory personnel (age 20-35 yr)	10	16	27	29	27	11
Normal nonlaboratory personnel (age 45-60 yr)	12	8	11	34	20	2
Cancer patients against cell line from similar tumor		9	12	18	19	5
Cancer patients against cell line from dissimilar tumor	5	5	12	15	14	12

*At least 10 individuals in each group.
†Results expressed as mean cytotoxicity above media control. Results of several experiments were combined.

It should also be noted in table 14 that, with 3 tumor lines, the average reactivity of cancer patients against cell lines of the same tumor type was higher than that of patients against a different tumor type. In many experiments, this form of comparison yielded "positive" results. However, if specific reactivity was defined as lysis significantly higher than any of the controls in the experiment, few positive results were seen (table 15).

TABLE 15.—*Summary of reactivity of cancer patients against cell lines of same tumor type*

Carcinoma	Patients		Number of tests in which patients had activity significantly higher than that in any of controls
	Number	Number of tests	
Colon	7	10	0 (against line 373)
Lung	4	5	1 (against line 2563)
Breast	17	19	4 (against at least 2 of the 3 breast tumor lines)

DISCUSSION

The ^{125}IUDR method for measuing cell-mediated cytotoxicity was modified and used for the present study because of several potential advantages. It is a technically simple, objective, and reproducible assay in which the isotope is incorporated exclusively into target cell DNA. Isotope is released only upon death and lysis of the cell, with almost no reutilization by unlabeled cells (*32–34*). The assay measures only cytotoxicity and not a combination of cytotoxicity, growth inhibition, or loss of adherence, as is measured by some of the other assays for cell-mediated immunity [e.g. (*2, 3*)]. Using trypsin seemed to add some sensitivity to the method in the animal studies, but it is not yet clear how useful trypsin will be with the human cell lines.

This assay also has some problems. Both radiation and chemical toxicity of ^{125}IUDR is well recognized (*35–37*). (A complete description of its toxicity and the kinetics of labeling in vitro is in preparation.) However, toxicity was not a major problem in the present studies and could be controlled by inclusion of a media control. Therefore, although proliferation may be inhibited after one replication cycle (*36*), the level of toxcity over the 3 days of this assay was acceptable.

The major problem we encountered was what represents an adequate control. This problem does not appear to be unique to the ^{125}IUDR assay, but rather exists with most of the in vitro cellular assays. Even in syngeneic animal tumor systems, the use of normal lymphocytes as controls may not be satisfactory. Studies in our laboratory with virus-induced lymphomas of rats and mice indicate that the lymphocytes of many normal animals have specific cytotoxic activity against tumor cells (*38*). This probably accounts, at least in part, for the observations in the present study that lymphocytes of normal rats produced higher lysis than those in the media control (table 5).

In human studies, the nature of the cytotoxic effects of normal lymphocytes is more difficult to define. With the ^{51}Cr-release assay, percent lysis significantly above the "autologous" cell control seems to occur only with previous sensitization (*31, 39*). Yet, many normal individuals have reactivity against leukemia-associated antigens (*39*) and against lymphoid cell lines (*31*). Some reactivity of normal controls has also been seen with colony inhibition or microcytotoxicity assays (*40–43*). Despite these scattered observations, little attention has been given to this problem, and most investigators still use the results of one or more normal controls as the baseline in their experiments. In our studies, when a number of normal individuals were tested against the human tumor lines, a considerable range of reactivity was seen. Younger individuals, who worked in laboratories, had generally higher activity. If age-matched controls were used as a baseline, more tests with patients would have been defined as positive.

The method of separation of lymphocytes from the peripheral blood may affect the results (*see* remarks in discussion period). Cells prepared on Ficoll-Hypaque gradients may produce more lysis than those purified on a nylon column. However, that would not completely explain the differences in control groups noted here, since all lymphocytes were prepared by the same method.

Another issue to be mentioned is the type of target cell used in the assays. Many previous studies have used cells in culture for only <2 months [e.g. (*3*)]. For our studies, we elected to use established tissue-culture lines. These lines offer the advantages of ready availability of the same target cells over an extended period and of more extensive characterization of the cells. It will be necessary to determine, however, whether more "nonspecific" results are obtained with these long-passaged cells.

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^{51}Chromium-Release Assay for Cell-Mediated Cytotoxicity of Human Leukemia and Lymphoid Tissue-Culture Cells [1, 2]

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SUMMARY—Cellular immune reactions to antigens associated with human leukemia were studied by a lymphocyte cytotoxicity assay employing the release of ^{51}chromium (^{51}Cr) from labeled cells of leukemic patients. Low-grade, but significantly positive, cellular cytotoxicity was obtained by the reaction of autologous or allogeneic lymphocytes from patients and from normal adult donors against cells from leukemic patients. Similar reactivity was not found against target cells from normal donors. The results suggest that human leukemia cells possess tumor-associated antigens. Studies to establish immunologic specificity of the positive reactions were technically difficult because of the low reactivity seen and because of difficult logistic problems in repeating the tests. Therefore, to study a variety of parameters that might help establish the specificity of the assay in human systems, human lymphoid cell lines derived from patients with tumors and from normal individuals were used as targets. Many of these (including F-265 and NC37) derived from normal lymphocytes were highly susceptible to lysis by lymphocytes of most normal donors. Agents, such as X-irradiation, actinomycin D, 3'5' cyclic-AMP, and concanavalin A, blocked either completely or partially the cytolytic activity of lymphocytes against F-265 cells. Target cell inhibition tests revealed that addition of graded doses of unlabeled F-265 cells always inhibited ^{51}Cr release from labeled F-265 cells. Similarly, inhibition of cytotoxicity of F-265 was also inhibited by some other cultured lymphoid cells including NC37, but was not inhibited by at least one other cultured lymphoid cell line (Stuck). Normal human lymphocytes stimulated in vitro by phytohemagglutinin were neither effective as inhibitor cells in the F-265 system nor were they directly lysed by lymphocytes of normal individuals. Finally, F-265 cells grown in media containing human sera instead of fetal bovine serum were effective targets in the assay. The results suggest that true cell-mediated immunologic reactions cause the lysis of human tissue-culture lymphoid cells by normal donor lymphocytes. The relevant antigen(s) appears neither to be "blast" associated nor to be related to antigens of fetal bovine serum. Hopefully, this system will be a useful model for defining the specificity of the cellular reactivity against leukemic cells.
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STUDIES with a variety of in vitro cellular immune assays using leukemic blast cells (*1–7*) and in vivo delayed-hypersensitivity reactions using leukemia cell extracts (*6–8*) indicate that human leukemia cells possess tumor-associated

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antigens. With one such assay, lymphocyte cytotoxicity with the use of ^{51}chromium (^{51}Cr) release from labeled target cells, peripheral blood lymphocytes from autologous leukemic patients, or lymphocytes from allogeneic patients and normal donors were shown to have a cytolytic effect against target cells from patients with acute leukemia (*4, 6, 7*). Similarly, in studies of genetically identical twins, cells from the twin with leukemia had antigens not detectable on lymphoid cells of the normal twin (*5*). The results of some assays against fresh leukemia cells are presented here to illustrate this reactivity.

Studies of the immunologic specificity of positive reactions in the leukemia system have been fraught with technical problems. The observed cytotoxic reactivity was generally quite low. In addition, there have been major logistic difficulties in obtaining adequate numbers of cells from the leukemic patient on the several repeated occasions needed for the specificity studies. Therefore, human lymphoid cell cultures were employed as model targets instead of leukemia cells, since we have found that normal donor effector lymphocytes have reproducibly high killing activity against some of these lines.

MATERIALS AND METHODS

Patients with acute leukemia were studied at the Clinical Center of the National Institutes of Health. Blood specimens from relatives, normal identical twins, and from normal adults were also studied.

Leukemia blast target cells or lymphocytes to be used as attacker cells were collected from the peripheral blood of patients or normal donors by venipuncture or leukophoresis or from bone marrow as previously described (*5, 7*). Briefly, blood was collected in 5–20 U/ml of heparin (Upjohn) and bone marrow in 20 U/ml. After isolation of the lymphocytes by dextran sedimentation of the erythrocytes or on Ficoll-Hypaque gradients (*9*), the white blood cells were washed and suspended, approximately 1×10^7 cells/ml, in culture media. Sometimes assays were performed with fresh target cells within 18 hours of collection. However, in most instances, the target cells were suspended in medium containing 10% dimethyl sulfoxide, frozen at a rate of 1° C/minute, and stored in liquid nitrogen. With attacking lymphocytes, the fresh cells were usually used within 2–24 hours after collection.

Tissue Culture of Human Lymphoid Cells

The established human lymphoid cell lines, including those derived from normal lymphocytes such as NC37, F-265 (*10*) (which lack Epstein-Barr virus particles or related antigens), and Stuck (kindly provided by Dr. S. Green, National Naval Medical Center), were routinely cultured in RPMI 1640 medium (Grand Island Biological Co.) supplemented with glutamine 2 mm and with 20% heat-inactivated (56° C for 30 min) fetal bovine serum or in some cases with 20% allogeneic normal human serum. Cultures were usually split every 3 days and were seeded at 3×10^5/ml. In some experiments, phytohemagglutinin M (PHA) (Difco, 6.8 μg/ml) was used to stimulate transformation of fresh normal peripheral lymphocytes in tissue culture for 72 hours.

Lymphocyte Cytotoxicity Assay

To prepare effector or attacker cells with high viability (>90%), lymphocytes were resuspended in Eagle's minimal essential medium with 10% heat-inactivated fetal bovine serum and 200 μg/ml carrageenan, a Cl complement inhibitor (*11*). Target cells (>75% viability) were labeled with ^{51}Cr after erythrocytes were removed with ammonium chloride Tris lysis. A 60:1 effector to target cell ratio was used, 3×10^6 effector lymphocytes were incubated with 5×10^4 ^{51}Cr-labeled cells from leukemia patients or tissue-cultured cells. To control for background ^{51}Cr release, labeled target cells were incubated alone (media control), with autologous unlabeled leukemia or tissue-cultured cells, or with normal donor peripheral blood lymphocytes which had been stored in liquid nitrogen (causing loss of most of their cytotoxic reactivity). Since a major concern involving human cellular immunity assays is the proper selection of control baseline values from which to deter-

mine positive and negative reactivities of test groups, we usually ran all of the above controls in most experiments. We have generally found that the unlabeled autologous control gave the lowest and most reproducible ^{51}Cr release; we, therefore, arbitrarily selected the autologous mixture as our baseline. Tests were always performed in quadruplicate. The mixtures were incubated in 13×100-mm tubes at a 20° angle for 4 hours at 37° C in a moist 10% CO_2 atmosphere on a Bellco rocker platform (6 rocks/min) to provide constant movement. After incubation, the tubes were centrifuged at $800 \times g$ for 10 minutes, and the counts per minute (cpm) of ^{51}Cr released into the supernatants were measured in an automatic gamma scintillation counter (Nuclear Chicago Corp.). The percent lysis of target cells was calculated relative to the maximum releasable ^{51}Cr, which was determined by freezing and thawing fractions of labeled target cells 4 times:

$$\% \text{ lysis} = \frac{\text{cpm } ^{51}\text{Cr released in experimental group}}{\text{cpm } ^{51}\text{Cr released from cells by 4} \times \text{ freeze-thaw}} \times 100.$$

Background release values ranged from approximately 5 to 30% lysis. Experimental results were expressed as corrected percent lysis, with the percent background release subtracted. Differences between experimental and control groups were analyzed for significance with the Student's t test. A test was considered positive if P was <0.05.

Inhibition of Cellular Cytotoxicity

a) Treatment with chemical agents.—Included in the incubation medium in some experiments was 10 μg actinomycin D to inhibit attacker lymphocyte reactivity. Similarly, in other experiments, 10^{-3} and 10^{-4}M butyryl 3′5′ cyclic-AMP (*12*) or 12–250 μg concanavalin A (Con A) (*13*) was added to the tubes before the 4-hour incubation. The viability of the lymphocytes was unimpaired following drug treatment, as assessed by trypan blue exclusion.

b) X-irradiation of effector cells.—In some experiments, normal donor lymphocytes were X-irradiated with either 500, 1000, or 5000 R and maintained at 4° C for 24 hours before use in lymphocyte cytotoxicity assays. The viability of all cell preparations was still usually 90% at the time of assay.

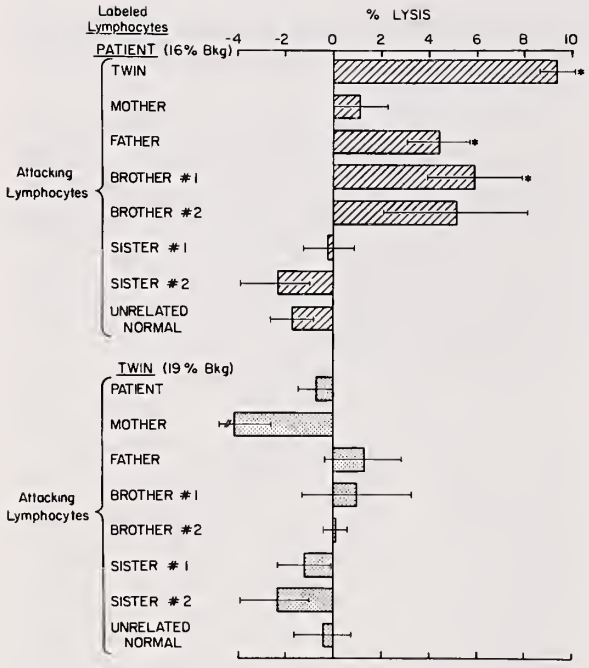
c) Target cell inhibition of cellular cytoxicity.—To define the specificity of the observed reactions, an assay for inhibition of lysis by unlabeled target cells was used (*14*). It is based on the concept that inhibitor cell populations containing antigens which the effector cells are presensitized against on labeled target cells cause a competitive inhibition of lysis. When added inhibitor cells lack the reactive antigen, lysis of the labeled target cells is not affected. These experiments employed 3×10^6 effector lymphocytes against 5×10^4 ^{51}Cr-labeled target cells. To this constant number of attacker and ^{51}Cr-labeled target cells were added various concentrations of autologous and allogeneic unlabeled target cells. The number of cells added included 1×10^6, 3×10^5, and 1×10^5, which corresponded to final attacker to target cell (A/T) ratios of 2.9/1, 8.6/1, and 20/1, respectively.

RESULTS

Identification of Leukemia-Associated Antigens by Lymphocyte Cytotoxicity Tests

Some of our studies to detect tumor-associated antigens on human leukemia cells were performed with sets of identical twins, family members, and unrelated normal individuals. Fresh peripheral leukemic blast cells from the patient were used as target cells. Peripheral lymphocytes from the patient's normal identical twin were also used as target cells to control for cytotoxicity due to variables or histocompatibility differences. The results presented in text-figure 1 of one representative identical twin study demonstrate that attacker lymphocytes from the normal twin, father, and 2 siblings reacted positively only against the patient's blast cells. The values of +4% lysis and greater were statistically significant ($P<0.05$), since ^{51}Cr-release counts of quadruplicate test samples were extremely close. None of the lymphocyte populations reacted with the target cells of the normal twin.

A more comprehensive summary of cellular cytotoxicity studies in human acute leukemia is presented in table 1, with results of reactions



TEXT-FIGURE 1.—Cytotoxic reactions of lymphocytes from family members against cells from leukemic and normal identical twins.

obtained with attacker cells from allogeneic ALL (acute lymphocytic leukemia) and AML (acute myelogenous leukemia) patients and normal individuals. The incidence of cytotoxic activity of all 3 populations of attacking cells was low against normal lymphocyte target cells. Conversely, a high percentage of reactions of attacker lymphocytes from either normal individuals or ALL and AML patients against ALL and AML blast target cells were positive. In addition, some positive reactions of normal lymphocytes against remission target cells were seen. The degree of lysis in these studies was similar to that shown in text-figure 1, with the positive reaction usually ranging from +4% to 10%. The results of these studies employing the lymphocyte cytotoxicity assay indicate that human leukemia blast cells and remission cells may possess leukemia-associated antigens.

Cellular Lymphocyte Cytotoxicity Studies of Human Tissue-Culture Systems

Cell inhibition studies to prove immunologic specificity of the positive reactions with human leukemic cells were technically difficult to do because of low cytotoxic reactivity of even the strongest positive tests (text-fig. 1). In addition fluctuation of reactivity of attacker populations of cells against leukemic target cells from one test to the next also complicated these studies. We had found during our studies that peripheral blood lymphocytes from most normal individuals were highly cytotoxic against several lymphoid tissue-culture lines derived from lymphomas or from normal peripheral lymphocytes. Therefore, we used these human lymphoid cultures as target cells in a model system to determine the feasibility of performing such studies of immunologic specificity in human leukemia.

Cellular Cytotoxic Reactivity Against Human Lymphoid Cell Cultures

The results in table 2 are representative of the cytotoxic effects of normal donor lymphocytes against target cells of cultures of human lymphoid cells. Results have been positive with cultures derived from tumor patients and also from normal individuals. F-265, NC37, and Stuck the cell lines used in this experiment, were all derived from normal lymphocytes. As shown, the 3 fresh donor peripheral blood lymphocyte populations readily killed high percentages of F-265 and NC37 cells but only weakly affected the Stuck target cells. These results also show that

TABLE 1.—*Lymphocyte cytotoxicity reactions against allogeneic target cells*

Target cells	Normal	Attacking lymphocytes	
		ALL patients	AML patients
Normal lymphocytes	5/220 (2.3)*	0/20 (0)	0/15 (0)
ALL blasts	55/117 (47)	9/23 (39)	4/15 (27)
ALL remission lymphocytes	15/79 (19)	0/5 (0)	0/2 (0)
AML blasts	40/95 (42)	6/14 (43)	6/25 (24)
AML remission lymphocytes	13/47 (28)	0	0/5 (0)

*Tests positive/total No. of tests; in parentheses are the percent positive reactions.

TABLE 2.—*Cytotoxic effect of normal donor lymphocytes against cultures of human lymphoid cells*

Attacker cells (3×10^6)	^{51}Cr-labeled target cells (5×10^4)		
	F-265	NC37	Stuck
Autologous	8.9%*	7.5%	10.1%
Donor A-peripheral blood lymphocytes	+5.9 (0.5)†	+10.3 (0.6)	+2.3 (0.2)
Donor B-peripheral blood lymphocytes	+18.1 (0.3)	+33.6 (2.0)	+3.5 (0.3)
Donor C-peripheral blood lymphocytes	+11.5 (0.6)	+24.1 (0.6)	+3.0 (0.2)
Frozen donor	+0.7 (0.5)	+0.9 (0.6)	+1.4 (0.3)

*Percent of "baseline" release of ^{51}Cr during 4 hours' incubation of labeled cells with unlabeled target cells used in place of attacker cells. A/T ratio = 60/1.

†Percent release of ^{51}Cr above the autologous values, in parentheses is given mean SE.

attacking lymphocytes from the same donor varied markedly in their ability to kill the 3 different target cells.

Reproducibility of Lysis of Lymphoid Tissue-Culture Target Cells

Table 3 presents the reproducibility of the lymphocyte cytotoxicity assay using ^{51}Cr-labeled F-265 target cells and effector peripheral blood lymphocytes from 3 normal individuals collected on 3 different occasions over a 21-day period. The results show that the cytotoxicity of lymphocytes from the first donor against F-265 cells was remarkedly close in all 3 tests. The cytotoxicity of the other 2 lymphocyte donors against F-265 fluctuated somewhat.

TABLE 3.—*Reproducibility of cellular lymphocyte cytotoxicity of normal donor lymphocytes against F-265 targets*

Attacker cells	Day	^{51}Cr-labeled F-265 target cells
Donor #1	0	27.7 (1.0)*
(Peripheral	7	27.0 (2.9)
lymphocytes)	21	27.0 (1.0)
Donor #2	0	16.3 (0.4)
(Peripheral	7	10.0 (0.4)
lymphocytes	21	12.1 (0.7)
Donor #3	0	21.5 (1.0)
(Peripheral	7	24.6 (0.8)
lymphocytes)	21	28.0 (1.2)

*Percent lysis above autologous baseline background; in parentheses is mean SE.

Characteristics of Normal Donor Lymphocyte Reactivity Against F-265 Cells

Tables 4 and 5 and text-figure 2 present studies on the characteristics of the cytotoxic reactivity of normal donor lymphocytes against tissue-culture F-265 target cells.

TABLE 4.—*Effect of X-irradiation on the cytotoxic activity of normal donor lymphocytes against F-265*

Attacker cells	^{51}Cr-labeled F-265 target cells
Autologous	7.2%
Donor peripheral lymphocytes	
Non-X-irradiated	15.0% (1.0)*
500 R†	14.5% (0.6)
1000 R	0.9% (0.5)
5000 R	0.2% (0.5)

*Percent above baseline; in parentheses is the mean SE.

†Attacker cells were X-irradiated, stored at 4° C, and used 24 hours later along with non-X-irradiated peripheral lymphocytes.

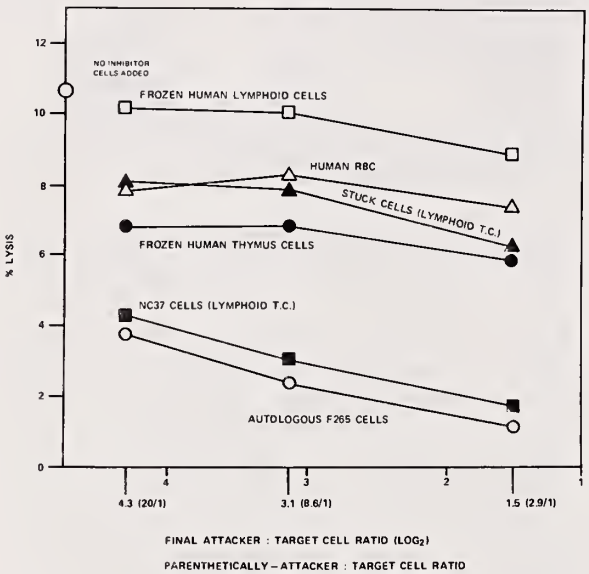
Effects of X-irradiation on cellular cytotoxic reactivity.—The results in table 4 show that, whereas 500 R X-irradiation of effector lymphocytes 24 hours prior to testing did not inhibit their activity against F-265 target cells, portions of these attacker cells exposed to 1000 R or 5000 R were markedly inhibited.

Effect of aging, freezing, drugs, and other agents on effector cell reactivity.—The results in table 5 show the effect of aging at 4° C, freezing to liquid nitrogen temperature, and drugs on the ability of normal lymphocytes to kill F-265 target cells. Low-temperature freezing of effector lymphocytes destroyed most of their reactivity against labeled F-265 cells. Similarly, aging the lymphocytes at 4° C for >48–72 hours also markedly reduced their reactivity, even though these preparations still remained highly viable at 96 hours. Storing the lymphocytes at 4° C for >48–72 hours also markedly reduced their reactivity, even though these preparations still remained highly viable at 96 hours. Storing the lymphocytes at 4° C for 24 hours did not inhibit their cytotoxic activity. In fact, as shown, the reactivity of the lymphocytes incubated for 24 hours appeared somewhat higher than that of nonincubated lymphocytes,

TABLE 5.—*Effect of aging, freezing, immunosuppressive drugs, and other inhibitory agents on ability of normal lymphocytes to lyse F-265 cells*

Treatment	Percent lysis produced by		Percent inhibition of lysis
	Treated cells	Untreated cells	
Viable cell freezing	3.4 (0.4)*	22.5 (0.7)	85
Actinomycin D (10 μg)	19.5 (1.2)	33.4 (1.4)	42
Butyryl cyclic AMP, 10^{-3}M	0.7 (0.6)	11.7 (0.6)	94
Butyryl cyclic AMP, 10^{-4}M	5.9 (0.5)	11.7 (0.6)	50
Con A, 250 μg	3.8 (1.0)	16.5 (1.0)	77
Con A, 100 μg	10.1 (1.7)	16.5 (1.0)	41
Aging at 4° C, 0 hrs (fresh)	20.2 (1.4)		—
Aging at 4° C, 24 hr	29.0 (1.6)		0
Aging at 4° C, 48 hr	14.4 (1.1)		29
Aging at 4° C, 72 hr	5.8 (0.9)		71
Aging at 4° C, 96 hr	4.7 (1.5)		77

*Percent lysis above baseline; in parentheses is the mean SE.



TEXT-FIGURE 2.—Inhibition of cellular cytotoxicity of ^{51}Cr-labeled F-265 cells by unlabeled target cells with the use of normal lymphocytes attackers.

an effect we have often observed. A concentration of 10 μg of actinomycin D was somewhat inhibitory in this system. Both butyryl cyclic AMP and Con A markedly inhibited normal lymphocyte reactivity against F-265 cells, although the effector cells remained highly viable.

Inhibition of cellular cytoxicity by unlabeled target cells.—Text-figure 2 presents the results of a cell inhibition experiment in which 3×10^6 normal donor lymphocytes were used against 5×10^4 ^{51}Cr-labeled F-265 target cells. To this usual reaction mixture, we also added various concentrations of unlabeled F-265 inhibitor cells. We also added the same concentrations of unlabeled NC37 and Stuck tissue-culture cells, human red blood cells, frozen human thymus, and frozen human lymphocytes as inhibitor cells. Both the unlabeled autologous F-265 and allogeneic NC37 cells markedly reduced the lysis of ^{51}Cr-labeled F-265 cells by normal donor lymphocytes, whereas none of the other unlabeled cells, including Stuck, appreciably blocked the cytotoxic reactivity. The curves show that the degree of inhibition depended on cell dose. These results indicate that F-265 and NC37 cells share antigens not detectable on the Stuck cell line nor on the normal human cells. Loss of antigenicity by freezing of the normal cells is not a likely explanation for the negative results, since comparable freezing of F-265 inhibitor cells did not affect their inhibitory capacity.

Use of PHA-Stimulated Lymphocytes as Targets in Cellular Cytoxicity Tests

To determine whether the antigen responsible for the cellular cytotoxicity reactivity might be related to the blastlike state of differentiation of the normal human lymphoid cultures, 72-hour, PHA-stimulated, normal peripheral blood lymphocytes were employed as target cells. Table 6 demonstrates that, although 40% of the PHA-stimulated lymphocytes were undergoing blastogenesis, they were not lysed by the normal lymphocytes. PHA-stimulated lymphocytes were

TABLE 6.—*Use of PHA-stimulated, normal donor lymphocytes as targets in cellular lymphocyte cytotoxicity assays**

Attacking lymphocytes	^{51}Cr-labeled target cells			
	Fresh lymphocytes	Cultured lymphocytes		
		Without PHA	With PHA	F-265
Autologous	9.9%	8.3%	8.7%	15.1%
Donor #1	0.8% (0.8)†	1.2% (0.6)	−1.9% (0.7)	30.1% (1.2)
Donor #2	0 (0.5)	−0.5% (0.6)	−1.2% (0.6)	21.9% (0.6)

*6.8 μg/ml of PHA was added to the media, and the cells were incubated at 37° C for 72 hours. Approximately 40% of the lymphocytes were stimulated to blastogenesis.

†Percent lysis above baseline; in parentheses is the mean SE.

also not active in inhibiting the cellular cytotoxicity of normal lymphocytes against F-265 targets (data not shown). Thus transformation of normal lymphocytes into blasts by PHA did not produce detectable amounts of the tissue-culture cell antigen.

Use of F-265 Target Cells Which Had Been Grown In Human Serum

Since the lymphoid cell cultures were routinely grown in 20% fetal bovine serum, the question arose as to whether the reactivity of normal lymphocytes was against bovine serum antigens bound to the F-265 cells. F-265 cells were, therefore, passaged for 5 generations in heat-inactivated (56° C for 30 min), normal human serum before use as target cells in lymphocyte cytotoxicity tests. The results with F-265 cells grown in human serum and with those grown in fetal bovine serum are presented in table 7. The reactivity of attacking lymphocytes was somewhat higher against the F-265 cells which had been grown in human serum. Thus, the reactivity of normal donor lymphocytes against cultured F-265 cells does not appear to be in response to fetal bovine serum antigens bound to their surface during culture.

TABLE 7.—*Use of F-265 cells grown in fetal bovine serum and human serum as targets in lymphocyte cytotoxicity assays*

Attacking lymphocytes	^{51}Cr-labeled F-265 target cells	
	Human serum*	Fetal calf serum
Autologous	17.8%	24.0%
Donor #1	36.1% (1.2)†	21.1% (1.8)
Donor #2	29.3% (0.8)	18.2% (2.1)
Donor #3	28.8% (0.8)	22.7% (1.9)

*F-265 cells were grown for 5 generations (a period of 1 month) in 20% human serum.

†Percent lysis above baseline; in parentheses is mean SE.

DISCUSSION

^{51}Cr-release cell-mediated cytotoxicity against human leukemia target cells was demonstrated in this study indicating that these cells may possess leukemia-associated antigens. These results corroborate earlier reports (*4–6*) of low-grade, significantly positive cellular immune reactivity against target cells from leukemia patients, employing attacking lymphocytes from patients and from normal healthy individuals. Again, in the present studies, some normal individuals gave positive cytotoxicity reactions against both patient leukemic and remission cells. The nature of the expressed antigens on the leukemic and remission cells is not yet known. However, there are some suggestions that the antigens of the 2 cell types may differ, since variations in patterns of reactivities have been found.

It was technically difficult to define the nature of the normal donor lymphocyte reactivity and the specificity of observed reactions of leukemia cells because of logistic problems in repeating the tests and generally quite low cytotoxic reactivity. Therefore, for specificity studies presented here, human lymphoid cell cultures were employed as model targets instead of leukemia cells, since we found that normal donor effector lymphocytes have a reproducible, high killing activity against some of these lines in our lymphocyte cytotoxicity assays. Our studies demonstrated that fresh donor peripheral blood lymphocytes readily killed high percentages of 2 human lymphoid cell lines (F-265 and NC37) but were only slightly reactive against a third

lymphoid cell line (Stuck). These results are similar to those of Holm and Hardy et al. (*15–17*) who reported active normal effector lymphocyte-killing activity against human cell lines. Holm (*15, 16*), however, could usually demonstrate reactivity against Chang liver cells by normal donor lymphocytes only in the presence of PHA, which appears to help bind or attract the lymphocytes to the target cells. Likewise, the system of Hardy et al. (*17*) required an in vitro prestimulation of normal attacker lymphocytes by X-irradiated populations of lymphoid cell lines, whereas our system did not require any in vitro presensitization.

A series of experiments designed to study inactivation or inhibition of lymphocyte cytotoxic lysis of our human lymphoid cell lines demonstrated many different effects on lymphocyte populations. For example, doses of 3′5′ cyclic AMP and Con A, previously reported to inhibit not only the cytotoxic activity of sensitized allogeneic C57BL lymphocytes against DBA/2 mastocytoma cells (*12*) but also the ability of PHA-stimulated human lymphocytes to destroy chicken erythrocytes (*13*), also strongly inhibited effector lymphocytes in our tissue-culture target cell system. Although the exact mechanism of these 2 phenomena is not known, it is believed that Con A may block lymphocyte effector sites necessary for the cytotoxic reaction or may kill the cell population essential for executing the lytic action (*13*), while elevated intracellular levels of cyclic AMP may modulate or control the lymphocyte-mediated cytolysis of target cells (*12*). Furthermore, the ability of lymphocytes from normal individuals to be reactive against tissue-cultured target cells was markedly impaired following slow freezing to liquid nitrogen temperatures or aging at 4° C in media for >48 hours, even though the cells remained highly viable. These findings, coupled with the loss of reactivity of effector lymphocytes following treatment with such agents as X-irradiation and actinomycin D, suggest that metabolically active cells are required to get optimum killing in the human lymphocyte cytotoxicity assay.

Finally, cell inhibition studies showed that the unlabeled autologous cell line as well as another allogeneic lymphoid cell line could inhibit cytotoxicity of ^{51}Cr-labeled target cells. Conversely, another tissue-cultured lymphoid cell line, previously demonstrated to be a poor target cell, was not active in blocking cytolysis of F-265. Similarly, inhibition was not obtained with human red blood cells, frozen human thymus, or frozen peripheral blood lymphocytes. These results indicate a specificity to the reaction, with inhibition only by cells sharing an antigen(s) with the ^{51}Cr-labeled target cells.

The nature of the relevant antigen system operative in the tissue-culture target cell cytolysis by normal donor lymphocytes is not yet known. However, several possibilities are as follows:

1) The antigen system is a "blast" antigen as earlier suggested in lymphocyte stimulation assays with leukemia cells (*2*) and autologous tissue-cultured lymphoblastoid cells (*18*). This possibility is unlikely in our studies, since PHA-stimulated, normal lymphocytes 72 hours in tissue culture were reactive neither as target cells nor inhibitor cells, even though approximately 40% of the cells were undergoing blastogenesis.

2) The antigen is a component of fetal bovine serum used to grow the human lymphoid cells in culture, which is bound to the surface. This possibility is also unlikely, since lymphoid cell lines grown for 5 generations in media supplemented with human serum instead of fetal bovine serum were highly reactive as target cells. Also, the lack of reactivity of the Stuck lymphoid cell line, grown in medium containing fetal bovine serum, would also argue against this possibility.

3) HL-A differences between target cells and effector lymphocytes cause the lysis of the cultured lymphoid cells by lymphocytes from normal healthy donors. This possibility is unlikely, since normal peripheral leukocytes can be stimulated by autologous tissue-culture lymphoblastoid cells in the lymphocyte stimulation assay (*18*) and cellular lymphocyte cytotoxicity of established lymphoid cell lines by autologous peripheral blood lymphocytes has been observed (*19*). HL-A typing of the lymphoid cultures and of the normal lymphocytes used in these studies is being conducted, however, to better clarify this possibility.

4) During the establishment of lymphocytes

in tissue culture, an antigen may be activated or induced which is usually in a weak or masked form in vivo. The expression of a new antigen or quantitatively more antigen in culture on some cell lines may make these lines susceptible to cytotoxicity by lymphocytes from normal individuals, if one postulates that a population of these effector lymphocytes has been presensitized and is immunologically committed to this antigen(s). Such a system may be part of the natural surveillance mechanism of immunologically competent hosts and may be strongly expressed in systems removed from the de novo situation. Similar observations of positive cytotoxic reactivity of normal human serum against cultures of human lymphoid cells have been reported (*20, 21*).

In summary, the data with the human lymphoid cell lines strongly suggest that viable and immunologically competent effector lymphocytes (presumably presensitized to relevant antigens) are necessary for cell-mediated cytotoxicity to occur. The inhibition of effector lymphocytes by various agents including X-irradiation and the target cell inhibition by autologous and allogeneic unlabeled target cells were similar to those seen with well-defined immunologic systems. Hopefully, similar investigations on the immunologic specificity of reactions in the human leukemia system, with the use of both effector and target cell inhibition, can now be more readily performed.

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Immunologic Studies in Acute Leukemia [1, 2]

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SUMMARY—Family members of 25 patients with acute leukemia were examined for cellular and humoral reactivity to leukemic cells. The ^{51}chromium (^{51}Cr)-release test, the indirect macrophage migration-inhibition factor (MIF) test, and the mixed leukocyte culture (MLC) test measured cellular immunity. Serum was tested for cytotoxicity as a measure of humoral immunity. ^{51}Cr-release and MIF tests were positive in about a quarter of the family members. Patients' sera always failed to block the reactions. Eight of nine HL-A-identical sibs were stimulated (MLC) by mitomycin C-treated leukemic cells. Serum from the father of a patient with acute lymphocytic leukemia (ALL) showed a complement-dependent cytotoxic reaction against the patient's tumor cells. This serum was not cytotoxic to normal lymphocytes of the two HL-A-identical sibs, their mother, nor the patient's lymphocytes when tested during remission. In addition to the father's serum, 2 sera from unrelated normal individuals were cytotoxic to the patient's tumor cells but not to the patient's remission lymphocytes. Further studies of these 3 sera revealed cytotoxicity to 6 of 6 ALL's and to 3 of 11 acute myelocytic leukemias, but no cytotoxicity to 2 acute monocytic leukemias, 2 leukolymphosarcomas, 4 ALL's with remission lymphocytes, and lymphocytes from >60 different normal individuals. Patients were also skin-tested with purified protein derivative of tuberculin, candida, trichophyton, streptokinase-streptodornase, mumps, and histoplasmin. When compared to a group of 20 normal controls, they were found to hyporeactive.—Natl Cancer Inst Monogr 37: 69–75, 1973.

ADVANCES in chemotherapy and supportive care over the past several years have led to remarkable gains in the treatment of certain forms of acute leukemia. Acute leukemia, however, is still incurable. The major emphasis in the management of this disease has been on intensive treatment with cytotoxic agents. These agents are designed to kill as many tumor cells as possible without irreparably damaging other vital cell systems, such as bone marrow, gastrointestinal tract, or liver.

A number of observations have encouraged clinicians to modify and broaden their therapeutic strategies against acute leukemia. These observations include the demonstration of tumor-specific antigens in animals and in man, the increasing recognition of the importance of host immune responses to tumors in animals, the documentation of the viral nature of certain animal tumors, and the collection of evidence for a possible viral etiology for certain human tumors. The toxicity of cytotoxic agents on immune function, the development of various antiviral agents, and the increased understanding of the principles of tumor im-

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munology and immunotherapy have become of paramount interest to the clinician.

These studies are preliminary attempts to measure immunologic responsiveness of patients and their family members to acute leukemia cells. We hope that the careful correlation of several in vitro tests of antitumor reactivity with the clinical courses of the patients will increase our understanding of the nature of immune responsiveness in neoplasia. This should, in turn, lead to the design of clinical trials of immunotherapy based on a better rationale than is currently available.

PATIENT POPULATION STUDIED

In general all patients were studied during their initial florid phase of acute leukemia (80–90% blasts in the peripheral blood) before receiving therapy. We studied 30 patients and their families (17 parents and 75 siblings). They were 12 patients with acute myelogenous leukemia (6 females and 6 males—mean age, 57.8 yr); 13 patients with acute myelomonocytic leukemia (4 females and 9 males—mean age, 50.0 yr); 1 male age 27 years with acute progranulocytic leukemia; and 4 patients with acute lymphocytic leukemia (ALL) (1 female and 3 males—mean age, 27 yr).

MATERIALS AND METHODS

Skin testing.—Antigens were injected (0.1 ml) into the skin of the back of each patient at admission. Reactions were read at 24 and 48 hours. Erythema and induration were measured in 2 perpendicular diameters. An induration 0.5×0.5 cm or greater was considered a positive response. The antigen battery consisted of: intermediate-strength purified protein derivative of tuberculin (PPD), 0.0001 mg or 5 tuberculin units/0.1 ml; mumps skin-test antigen; Varidase [streptokinase/streptodornase (SKSD) mixture, 40 U and 10 U, respectively]; *Candida albicans* extract, 1:100 dilution of full-strength antigen; *Trichophyton gypseum* extract, 1:30 dilution; and histoplasmin, 1:100 dilution. Skin testing with autologous tumor was performed by intradermal injection of a 0.1-cc suspension of 25×10^6 tumor cells (inactivated by incubation with mitomycin C, 75 μg/10^6 cells, 37° C, for 60 min, washed, and suspended in RPMI 1640 medium). Leukemic cells were incubated for various times up to 8 days and then cultured for 16 hours in tritiated thymidine; they showed no growth or thymidine uptake after mitomycin C treatment.

HL-A typing and testing for serum cytotoxic antibody activity.—These procedures were performed in triplicate cultures in Falcon plastic microtiter trays. A total of 1 ml (10^6 cells/cc) of cell suspension (nylon column-purified lymphocytes) was incubated with 2 μl of each typing serum or each unknown serum for 30 minutes at room temperature. The wash step, described in (*1*), removed excess antibody. The cells were then incubated for 1 hour at room temperature with 4 ml of rabbit complement. Trypan blue dye was added, and reactivity was determined by dye exclusion. A reaction was considered cytotoxic if at least 20% of the cells were killed.

Mixed leukocyte cultures (MLC).—One-way MLC were done with modifications of techniques described in (*2*). Heparinized venous blood was settled with plasmagel, and the leukocyte-rich plasma was extruded and centrifuged. Erythrocytes were lysed with Tris-NH_4Cl. Cells were cultured in RPMI 1640 tissue-culture medium, with streptomycin, penicillin, and 15% fetal calf serum (FCS). Stimulating cells were treated with mitomycin C (50 μg/1.5×10^6 cells) for 30 minutes at 37° C and washed. Cell counts were adjusted for lymphocyte content. Responding lymphocytes and stimulating lymphocytes or tumor cells were mixed in a 1:1 ratio, 0.75×10^6 cells of each, or a total of 1.5×10^6 cells/cc, each culture being 1 cc, in 16×100-mm test tubes. Triplicate cultures were held in a humidified 5% CO_2 incubator at 37° C. At about 100 hours of incubation, 1.0 μCi of tritiated thymidine (specific activity 1.9 Ci/mM) was added. Cultures were harvested 16 hours later, and DNA was precipitated on glass fiber filters with cold 5% trichloroacetic acid. Filters were dried with absolute ethanol, suspended in scintillation fluid, and counted in a beta scintillation counter. Controls were done of all combinations of each family member and patient being used as responding and stimulating (mitomycin-treated) cell sources, and all

cells were tested for ability to stimulate and to respond to an unrelated control and to phytohemagglutinin. Control tests with mitomycin C-treated cells alone showed essentially no—<50 counts per minute (cpm)—thymidine incorporation. Standard error among triplicate cultures was about ±10–15%. The MLC is known to vary from day to day in absolute counts because of technical variables; therefore, all tests and all appropriate controls on a given patient and his family were done simultaneously on a single day. Mean counts for triplicate cultures were calculated, and the stimulation index (SI)—cpm experimental lymphocyte mixture divided by cpm autologous lymphocyte mixture—was determined to compare different patients' families tested on different days. An SI of ≥2.0 was considered a significant response.

^{51}Chromium (^{51}Cr)-release cytotoxicity test.—Cell-mediated ^{51}Cr-release cytotoxicity testing was done essentially as described in (*3*). Lymphocytes from heparinized venous blood were purified on a Ficoll-Hypaque gradient, washed in Hanks' balanced salt solution with 6% heat-inactivated FCS, and suspended at 1.0×10^7 cells/cc in Eagle's minimal essential medium with 12% FCS. Target cells were leukemic blasts prepared similarly, to a concentration of 1.5×10^7 cells/cc. To 1 cc of target cell suspension was added 250 μCi of ^{51}Cr (0.25 ml of $Na_2\ ^{51}CrO_4$, specific activity 100–500 μCi/μg of sodium chromate), and the cells were incubated 45 minutes in a shaking 37° C water bath. Excess chromium was washed off, and target cells were suspended in Eagle's minimal essential medium with 12% FCS at a concentration of 2.0×10^6 cells/cc. Attacking cells (from family members or unrelated controls) and labeled target cells were set up in triplicate, at a 1:100 target to attacking cell ratio, in a total volume of 1.05 cc in plastic petri dishes. Cultures were incubated on a rocking platform at 37° C in a humidified 5% CO_2 atmosphere for 4 hours. Then the contents of the petri dishes were emptied into test tubes and centrifuged, and released radioactivity was ascertained in a gamma scintillation counter. The degree of cytotoxicity in the experimental cultures was expressed as a percent of the total release of ^{51}Cr from labeled, freeze-thawed, disrupted target cells. As a control for spontaneous leak, 1.0×10^5 labeled target cells were added to 1.0×10^7 unlabeled target cells and handled simultaneously with the experimental cultures. Spontaneous release of ^{51}Cr from the control tubes after 4 hours was approximately 12–16% of release from freeze-thawed labeled cells. Calculations for cytotoxicity were: percent lysis = cpm ^{51}Cr release from target cell lymphocyte mixture × 100, divided by cpm ^{51}Cr release from freeze-thawed cells. The percent lysis of the experimental test was compared to that of the control by the Student's *t* test. Significance was accepted at 5% which usually corresponded to a ≥5% release from the experimental than from the control (spontaneous release, as above) cultures. In table 3, results are expressed as number of family members whose lymphocytes caused ^{51}Cr release from labeled leukemic cells, divided by the number of such combinations tested.

Macrophage migration-inhibition factor (MIF) test.—MIF tests were done by modifications of techniques described in (*4*, *5*). Lymphocytes were purified from heparinized venous blood with a Ficoll-Hypaque gradient. Leukemic blasts (stimulating cells) and lymphocytes (responding cells) from each family member were mixed in RPMI 1640 tissue-culture medium, with 0.5% FCS, in a concentration of 2.5×10^6 cells/cc of each. Responding and stimulating cells were also cultured alone at 5.0×10^6/cc. Cultures were incubated in a humidified atmosphere of 37° C in 5% CO_2 for 72 hours. Supernatants were harvested every 24 hours and pooled. Supernatants from unmixed cultures were pooled together in the various test combinations as controls. After final harvesting, pooled supernatants were dialyzed against physiologic saline and lyophilized. Lyophilized supernatants were reconstituted at 10 times the original concentration in RPMI 1640 with 10% normal guinea-pig serum and tested for inhibition of guinea-pig peritoneal exudate (mineral oil induced) cells from capillary tubes in Sykes-Moore chambers. The area of migration was projected on paper and measured with a compensating planimeter. Experimental mixtures were those supernatants from cultures in which leukemic blasts and lymphocytes from family members were mixed together. Controls

were supernatants mixed from independently cultured cells, cultures of tumor plus lymphocytes from unrelated controls, and cultures from positive controls of family members or unrelated controls whose lymphocytes were mixed with antigens to which they had shown a response by skin testing. The mean of the area of migration of the experimental mixture was compared to that of the control (supernatants mixed from independently cultured cells) by the Student's t test, and significance was accepted at 5%. In table 3, results are shown as the number of family members with significant MIF when their lymphocytes were cultured with leukemic blasts, divided by the number of such cases tested.

RESULTS

Skin Testing

Table 1 shows the results of skin testing of 24 patients with acute leukemia and a control group of 20 unrelated normal volunteers. The data clearly indicate that patients with acute leukemia are often unresponsive to the antigens tested, i.e., are "anergic." Those not totally anergic tend to show a smaller number of positive responses than normal controls, but the incidence of response to a given antigen in this nonanergic leukemic group tends to parallel that of the control group. We found no delayed-hypersensitivity response to intradermally injected autologous tumor cells.

HL-A Typing and MLC

Of 75 siblings in the 30 families studied, 23 had HL-A-identical matches. Nine normal siblings were HL-A identical to leukemic patients. There was no evidence in the typing data for deletion or addition of HL-A antigens on the leukemic blasts. Table 2 summarizes our results with the MLC technique, which measured the stimulation of siblings by HL-A-identical normal sibling's lymphocytes and by HL-A-identical sibling's leukemic blasts. We will report the results of MLC testing in more detail in the future (*6*). Of 9 HL-A-identical siblings tested for response to leukemic blasts, only 1 failed to show an SI of ≥ 2.0. We saw no

TABLE 1.—*Skin testing in acute leukemia**

	Number of persons positive					
	PPD	Mumps	SKSD	*Candida*	*Trichophyton*	Histoplasmin
Control (20)	1	19	20	10	2	1
Acute leukemia (24)	1	8	11	2	1	2

	Number of persons in each reactivity score group†						
	0	1	2	3	4	5	6
Control (20)	0	1	8	9	1	1	0
Acute leukemia (24)	10	5	7	2	0	0	0

*All patients negative to skin testing with autologous tumor.
†Number of persons responding 0, 1, 2, etc., to the skin test antigens.

TABLE 2.—*Responses of HL-A-identical siblings to leukemic blasts (MLC)*

	Number of tests	Mean SI*	Standard error	P
Sibling response to normal HL-A-identical siblings	14	0.9	±0.099	<0.0005
Sibling response to HL-A-identical leukemic blasts	9	2.5	±0.300	

$$*SI = \frac{\text{cpm experimental leukocyte mixture}}{\text{cpm autologous leukocyte mixture}}.$$

responses in mixed cultures of normal HL-A-identical siblings. In 1 case of ALL, leukemic lymphoblasts stimulated an HL-A-identical sibling's lymphocytes. Patients' lymphocytes obtained during remission did not stimulate, but frozen, stored leukemic blasts continued to stimulate. Similar studies on other cases are in progress.

^{51}Cr-Release Cytotoxicity and MIF Tests

Table 3 summarizes the evidence for antitumor cellular immunity in family members studied with the ^{51}Cr and MIF tests. Cytotoxicity, indicated by ^{51}Cr release from leukemic blasts, was positive in 6 of 43 family members tested. Similarly, macrophage migration was inhibited in 6 of 34 MIF tests by supernatants obtained by the culture of family members' lymphocytes with leukemic blasts. Positive responses in 1 mother and 2 sisters in the series may have been due to presensitization to HL-A antigens caused by multiple pregnancies. In none of the positive tests did serum from the respective patients in relapse either blunt or block these positive responses; i.e., we found no evidence for "blocking" activity in patients' sera.

Cytotoxic Antibody

Sera from each patient and each family member were assayed for cytotoxicity to tumor cells of the patient and to lymphocytes of the other family members. We found 3 cytotoxic sera: 1 (M.B.) from the father of a patient with ALL, 1 from a brother of another patient with ALL, and 1 from a brother of the patient with acute progranulocytic leukemia. The latter 2 sera have not yet been characterized. The first sera (M.B.) and sera from 2 other unrelated normal individuals (N.N. and M.W.) appeared to have cytotoxic specificity for ALL cells, as reported in (7).

These 3 sera were tested against a number of freshly collected cells from other patients with different leukemias and against lymphocytes from normal donors. Results are in table 4. The one unequivocally positive cytotoxic reaction to other than ALL cells was that to the single case of acute progranulocytic leukemia in our series. From our investigations (including absorption studies) of normal lymphocytes from unrelated and normal family donors, and of lymphocytes from ALL in remission, we have tentatively concluded that these sera were detecting antigens primarily associated with ALL (7).

Recently we identified, but have not yet fully characterized their specificity, 3 additional sera with cytotoxicity to ALL cells: 1 from a boy in a 4-year clinical remission from ALL, 1 from his pediatrician, and 1 from another normal blood donor.

TABLE 4.—*Sera cytotoxic to ALL**

	M.B.†	N.N.†	M.W.†
ALL	6/6	7/7	6/6
ALL remission	0/4	0/4	0/4
Acute myelocytic leukemia‡	2/11	2/11	2/11
Acute progranulocyte leukemia	1/1	1/1	1/1
Acute monocytic leukemia	0/2	0/2	0/2
Leukolymphosarcoma	0/2	0/2	0/2
Normal lymphocytes	0/60	0/500	0/60

*All sera complement dependent for cytotoxicity.
†Activity against ALL not removed by absorption with ALL remission lymphocytes, nor family members' lymphocytes.
‡Reactivity was ± in these 2 cases.

DISCUSSION

We found rather marked cutaneous anergy in patients in the florid stage of acute leukemia. This is consistent with the observations of others that patients with a variety of neoplasms may display cutaneous anergy when their disease is

TABLE 3.—*Antitumor cellular immunity in family members**

Fathers		Mothers		Brothers		Sisters	
MIF	^{51}Cr	MIF	^{51}Cr	MIF	^{51}Cr	MIF	^{51}Cr
0/2	0/3	0/1	1/3	4/15	2/17	2/16	3/20

*Number of tests with positive response/No. tests done.

widespread and advanced. Future studies of cutaneous reactivity and in vitro studies of lymphocytes and sera of such patients done serially throughout their treatment courses and remission may provide further insight into the mechanisms and significance of these immune deficiencies. Preliminary work of Ragab and Cowan (*8*), for instance, indicated that a subpopulation of cells responsive in the MLC test may be separated from the peripheral blood of leukemic patients in which unseparated leukocytes showed little or no response. In addition, their data indicate that the lack of MLC response in unseparated leukocytes was not due to factors present in the leukemic sera.

In vitro humoral and cellular immunity to tumor cells in nonparous and nontransfused family members and other normal individuals has been shown in a variety of solid tumors (*9, 10*). In our studies, about a quarter of the family members showed some form of humoral (cytotoxic antibody) or cellular (^{51}Cr release or MIF positivity) immunity to leukemic cells. These data and those of others are compatible with, and strongly suggestive of, a viral etiology for these malignancies. Immunization by natural exposure to such putative oncogenic viruses could produce the type of results we observed in patients' relatives.

In these studies, 8 of 9 HL-A-identical siblings responded to leukemic blasts in MLC. The observation is in apparent conflict with the results reported by Halterman and Leventhal (*11*) and Rudolph et al. (*12*), but confirms the observations of Bach et al. (*13*). We cannot explain the discrepancy. Halterman and Leventhal primarily studied a pediatric-age leukemic population, while Rudolph et al. studied identical twins. Whether these differences from our sample are important is unknown.

The significance of positive reactivity in the MLC response is not readily apparent. At least 3 possibilities exist: 1) The leukemic blast cell surface expresses minor histocompatibility antigens in a way that stimulates MLC; 2) the leukemic blasts express blast or embryonic antigens not unique to leukemic cells; and 3) the leukemic cells possess a leukemia-specific tumor antigen. None of these hypotheses can yet be proved. The results of our studies with cytotoxic antibody, considered with those of others (*11, 14, 15*), show that leukemic patients in remission can respond in MLC to autologous tumor cells and persuade us to favor the third possibility.

The discovery of cytotoxic antibody with apparent specificity for ALL cells needs further study and confirmation. The report of Fialkow et al. (*16*), of recurrence of ALL in the donor cells after a marrow transplant, and our preliminary serologic studies are consistent with an association of an infectious agent with acute leukemia.

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Quantitation of the Microassay for Cell-Mediated Immunity Through Electronic Image Analysis [1, 2]

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SUMMARY—Increased precision, speed, and objectivity in the counting of surviving target cells were accomplished by the application of image analysis and electronic counting to the microassay for cell-mediated immunity. Reproducibility and variability of cell counts by the same and different operators were studied; agreement was satisfactory. The quantitative approach that became possible was applied to problem areas in the test, such as position effects, differential multiplication of target cells, and volume and duration of the assay. The choice of a correct control to use in the test also became a problem when some normal individuals were found to react strongly and feeder effects were observed. A score was developed based on percent reduction from either medium controls or from negative lymphocyte tests with the maximum number of target cells.—Natl Cancer Inst Monogr 37: 77–84, 1973.

ASSAYS EMPLOYING cell-mediated immunity are being used increasingly in tumor and transplantation immunology because they are highly sensitive. The tests are quantitated by either counting of remaining target cells (*1, 2*) or by isotope uptake or release from target cells (*3–8*). A major difficulty in cell-mediated tests has been in dealing with the many factors contributing to variability, such as effector and target cells, culturing conditions, and termination and reading of the tests. With the application of electronic image analysis to counting of surviving target cells, it is now possible to study these factors in detail for tests such as the microassay for cell-mediated immunity (MCI) which measures target cell destruction by adherence to plastic. If prepared correctly, test plates can be read quickly, objectively, and precisely. This report describes the precision possible through electronic counting and our attempts, through the application of a quantitative approach, to achieve better reproducibility in the test.

MATERIALS AND METHODS

Target cells.—Cells from human tumors were cultured and tested in minimal essential medium with 20% fetal bovine serum and antibiotics. Both short-term and established lines were used. Test cells were plated into the wells of a microtest plate (Falcon 3034) and incubated for desired periods. To prepare the tray for counting, the wells were washed with Hanks' solution to remove debris and unattached cells. Adhering cells were then fixed in 50% acetone-ethanol, stained with Giemsa solution, washed, and dried.

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Electronic counting.—The prepared trays were placed in the Quantimet 720 (Imanco), the detector levels were adjusted for contrast, and the image was focused on the screen. We made certain that each cell was designated by a marker. The number was recorded by teletype. Besides focusing of the microscope, adjustments on the image analyzer were for sizing and darkness of the objects to be counted. The mode was set for full feature rather than end count to avoid recounting odd-shaped cells.

MCI.—The test has been described previously (*2, 9*). In each well, 100–300 target cells were plated in the microtest plate and allowed to adhere. Lymphocyte samples with <10% granulocyte contamination were isolated from peripheral blood of patients and normal individuals. Four dilutions starting with 50,000 lymphocytes were overlaid on the target cells. After the cells had settled to the bottom of the wells, the plates were flooded with 5–7 ml medium and the test mixture was incubated for 2 days. The test was terminated as described above.

Scoring.—The percent target cell reduction for titrations was designated as a, b, c, and d for lymphocyte numbers of 50,000, 25,000, 12,500, and 6,250, respectively, per well. MCI score = 1a + 2b + 3c + 4d.

RESULTS

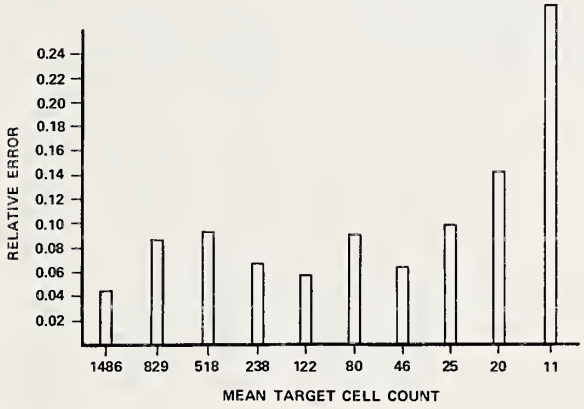
To determine the precision of counting target cells with the Quantimet 720, HeLa cells were titrated by 1:2 dilutions for 10 steps. For each dilution, 10-μl samples were plated across the 6 wells in each column of the microtest plate. The titration plate was counted by image analysis by 7 different operators. We determined a relative error for each titration or column by dividing the standard error with the mean number of target cells. Table 1 shows the count determined for the complete plate by 1 person, a comparison of a single row counted by 7 different operators, and the same row read by 1 person 6 different times. The average relative errors for the 10 columns in the above-

TABLE 1.—*Electronic counting of a plate with titered dilutions of target cells*

			Number of target cells—column									
Plate	Operator	Row	1	2	3	4	5	6	7	8	9	10
5168	J	F	1345	593	457	242	119	70	54	16	27	10
		E	1376	957	472	249	127	76	46	25	11	10
		D	1501	992	582	275	150	46	43	26	20	26
		C	1360	885	711	200	124	95	38	29	12	9
		B	1561	869	676	197	148	101	53	32	10	5
		A	1393	618	439	277	106	84	50	33	21	10
		Mean	1423	819	556	240	129	79	47	27	17	12
	Standard error		35.9	70.3	48.3	14.3	7.0	8.1	2.5	2.5	2.8	3.0
	Relative error		0.025	0.085	0.086	0.058	0.054	0.101	0.053	0.093	0.166	0.255
5168	B	B	1561	869	676	197	148	101	53	32	10	5
	C	B	1754	834	435	235	137	99	42	28	13	8
	H	B	1747	715	688	253	154	104	49	29	19	9
	J	B	1555	887	678	204	105	102	53	28	12	9
	U	B	1721	922	679	249	150	102	53	26	13	11
	P	B	1684	828	595	237	147	103	53	32	12	8
	X	B	1400	629	455	217	106	75	51	18	28	13
		Mean	1632	812	601	227	135	98	52	28	15	9
	Standard error		49.8	39.3	42.1	8.3	7.9	3.9	0.6	1.8	2.4	1.0
	Relative error		0.030	0.048	0.070	0.037	0.059	0.038	0.011	0.064	0.160	0.111
5168	J	F	1244	646	400	231	118	72	53	17	30	22
		F	1242	667	448	230	121	63	53	17	30	24
		F	1226	663	431	219	114	69	54	18	29	25
		F	1181	621	442	220	113	75	59	14	29	21
		F	1339	702	431	242	108	68	55	20	26	22
		F	1365	671	458	229	120	77	53	21	30	23
		Mean	1266	662	435	229	116	71	55	18	29	23
	Standard error		28.9	11.0	8.2	3.4	2.0	2.1	1.0	1.0	0.6	0.6
	Relative error		0.022	0.016	0.019	0.015	0.017	0.029	0.018	0.056	0.022	0.026

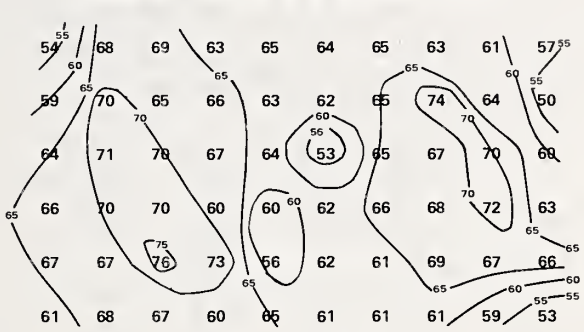
mentioned groups were 0.098, 0.063, and 0.024, respectively. Counting was less precise when compared between operators than by a single person, but was better than the variability found in the number of target cells plated among replicates. Counting error from the Quantimet 720 is not a major factor in the test.

The relative errors were also determined for each operator reading the titration tray. A mean relative error for each titration was drawn from these results (text-fig. 1). Since counting by image analysis was most precise in the region of 50–300 cells, we tried to keep target cell levels within this range.



TEXT-FIGURE 1.—Relative counting error for a titration plate. *Each bar* represents the mean relative error for 7 operators reading 1 titration on a plate.

Very early during the use of microtest plates with cultured cells, it was observed that cells did not grow as well along the edges and especially in the corners of the plate. With automated counting, this problem could be looked at more precisely and objectively. A total of 19 different short-term and established cultured cells, including epithelioid, fibroblast, and mixed cells, were added to separate plates in numbers predetermined to give 100–300 cells per plate; they were incubated for the usual test period. To ascertain the effect of position on the plate, the target cell numbers from the 19 plates were averaged for each well after the numbers were corrected to equalize the variability found between plates by the formula, 100 $(\chi^{0.2}-2)$. Text-figure 2 shows the contour effect according to the position on the plate.



TEXT-FIGURE 2.—Corrected average of target cells for 19 uniformity plates. Data plotted are the average of $(\chi^{0.2}-2) \times 100$. The standard error of the average is 3.3. Differences among wells are statistically significant at $P<0.0005$.

To equalize in the simplest way position effects for all lymphocyte samples tested, the microtest plate was divided into exact quadrants (I–IV). Titrations of lymphocytes were started in the inner column and then progressed outward; the outside wells were retained for medium controls. Table 2 shows that, in the average number of target cells for each triplicate tested, the number was never less than the average for all controls. Results of target cell reduction by effector lymphocytes from patients and normal individuals are also shown for comparison.

Differential growth of the cells in the microtest plate accounts for part of the position effect. Cells were seeded into replicate plates, half of which were flooded after the cultured cells had attached themselves. One plate from each group was fixed and stained daily for 4 days. The results (text-fig. 3) show the daily range for each column. Target cell numbers increased daily as did the variability between wells as reflected in the wider range for each column. Growth tended to be better in the center of the plate.

Tests with lymphocytes were also carried out in different volumes for 1–4 days. Twelve plates testing 2 individuals in 2 quadrants were started, with the only difference being the volume of media used. One plate each of the tests performed in 10 and 15 μl and with flooded plates was terminated each day for 4 days. The results are given in table 3. Although the same relative differences could be detected from the 1st day,

TABLE 2.—*Testing in quadrants on the microtest plate*

A. Uniformity plates (no lymphocytes added)

Corrected average for 19 plates

	I. Titrations					II. Titrations				
	C	4	3	2	1	1	2	3	4	C
	54	68	69	63	65	64	65	63	61	57
	59	70	65	66	63	62	65	74	64	50
	64	71	70	67	64	53	65	67	70	60
Mean	59.0	69.7	68.0	65.3	64.0	59.7	65.0	68.0	65.0	55.7
	III					IV				
	66	70	70	69	60	62	66	68	72	63
	67	67	76	73	56	62	61	69	67	65
	61	68	67	60	65	61	61	61	59	53
Mean	64.7	68.3	71.0	67.3	60.3	61.6	62.6	66.0	66.0	60.3

Control average 59.925 Correction is 100 ($\chi^{0.2}-2$)

B. Test on target cell 416 (breast cancer)

Target cells per well

	I					II				
	C	4	3	2	1	1	2	3	4	C
	122	198	164	164	108	84	102	145	180	124
	201	183	140	137	69	51	70	150	121	150
	215	157	138	125	67	32	94	136	140	213
Mean	181.3	179.3	147.3	142.0	81.3	55.7	88.7	143.7	147.0	165.7
	III					IV				
	211	189	201	157	96	73	113	135	172	172
	168	165	172	151	71	60	124	140	161	167
	138	138	136	134	59	55	122	157	154	86
Mean	175.7	164.0	169.7	147.3	75.3	62.7	119.7	144.0	162.3	141.7

Control average 164.0

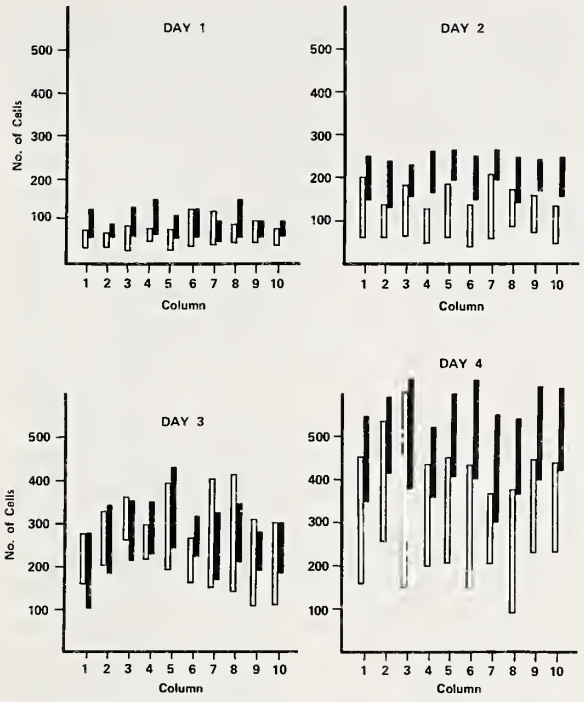
Type of cancer		Percent reduction 1	2	3	4	MCI score
I	Normal	51	13	10	0	107
II	Breast	65	46	13	10	236
III	Nasopharynx	54	10	0	0	74
IV	Uterus	62	26	12	1	154

target cell reduction continued to increase until the 3d day. The different volumes tested varied only slightly but flooded trays seemed to give the most sensitive results.

TABLE 3.—*Varying volume and time in the MCI*

Duration of test (days)	Person tested	MCI scores on target cell 470 10 ml	15 ml	Flooded
1	A	133	166	291
	B	42	67	79
2	A	452	359	471
	B	202	279	301
3	A	545	532	659
	B	473	318	461
4	A	529	664	595
	B	380	312	256

To monitor patients for changes in reactivity, it is necessary to use stable controls. Most studies to date have used tests with normal individuals as controls (*1, 10, 11*). Table 4 shows the results of tests with lymphocytes from 12 healthy donors on 19 target cells, several of these being tested on the same plate. Two observations had direct implications to testing. Established lines were more sensitive to cellular effects even from normal persons than were short-term cultures. There were very strong positive reactors even among the normal group, and considerable variability



TEXT-FIGURE 3.—Differential growth of target cells in the microtest plate. *Each bar* represents the range in the number of cells per column. *Light bars* were plates with cells incubated in 10 μl medium. *Shaded bars* were plates flooded with 5–7 ml medium after the cells had adhered.

was observed among others. Thus the reactivity of the patient tested cannot be accurately measured with the use of normal controls, since the strength of the reaction depends on the normal individual selected for comparison. If medium alone with no lymphocytes added was used as a negative control, problems were also encountered. The average number of target cells in the medium controls was compared with cell numbers in the titration with the lowest ratio of lymphocytes in 331 test plates. In 79 (23%) of the tests, target cell survival in the test exceeded by at least 10% the number observed in the control wells. Increased survival was attributed to a feeder effect by lymphocytes and partially to differential growth and the position of the control on the end of the plate. However, a lower number in the controls gives a more conservative estimate of cell-mediated target cell reduction. No correction was needed for most tests in determining the percent reduction or the MCI score, but for those exhibiting the feeder effect, the mean for the final dilution of negative lymphocytes was substituted.

The value of a test measuring a patient's degree of immunologic sensitization against tumor-associated antigens depends on the test's reproducibility. Ten patients were tested on the same target cell at least twice from different bleedings. The results are shown in table 5 with the percent reduction for each dilution of lymphocytes, the titer at 20% target cell reduction, the sum of the percentage reduction for the 4 dilutions, and the MCI score. Including the 3 tests of 1 patient on target cell 414, 20 comparisons were possible. On examination of the titer, 5 differed by 2 dilutions, another 6 by 1 dilution, and 9 comparisons agreed. When the repeated testing was

TABLE 4.—*Target cell reduction by lymphocytes from normal individuals*

Series		Person tested	MCI scores						
1	Target cell		370*	373*	414				
		42602	101	4	20				
		42614	148	655	83				
		42617	105	83	170				
2	Target cell		224*	472	458	425	388	380	
		42677	285	8	0	14	24	47	
		42755	242	210	2	14	46	126	
		42756	468	496	147	43	432	422	
3	Target cell		123*	414	419				
		42802	26	45	2				
		42800	52	0	7				
4	Target cell		388	438	458	496*	124*	157*	250
		42811	125	15	0	701	523	138	29
		42812	240	15	0	830	0	0	0
5	Target cell		419	455	438	414	413	388	
		42849	45	0	0	0	31	33	
		42850	17	0	0	0	15	0	

*Established line.

compared on the basis of the sum of the percentage reduction for the 4 titrations, 8 of 20 agreed within 10% and 13 of 20 within the 20% of the total scale. For the MCI score, this was 11 of 20 within 10% and 14 of 20 within 20% of the scale. Although the exact scores cannot be reproduced, strong and weak reactors can be distinguished and it was believed that valid comparisons could be made between test plates and tests performed on different days.

TABLE 5.—*Reproducibility of MCI results from rebleedings of the same patient*

Target cell	Sample No.	Percent target cell reduction				Titer	Sum	MCI score
		1	2	3	4			
463	42599	43	25	36	0	3	104	201
	41656	83	78	54	52	4	267	609
496	42599	80	42	10	0	2	132	194
	42565	96	82	34	10	3	222	402
462	42599	41	40	38	0	3	119	235
	42680	92	89	37	20	3	238	461
425	42599	14	14	21	0	–	49	105
	42680	42	38	12	0	2	92	154
373	42491	54	30	3	0	2	87	123
	42561	96	82	65	62	4	305	703
413	42491	16	0	0	0	0	16	16
	42561	74	41	0	0	2	115	156
463	42491	75	73	49	31	4	228	492
	42561	96	92	69	51	4	308	691
287	42354	41	29	12	5	2	87	155
	42978	85	55	57	36	4	233	510
414	42355	1	0	0	0	0	1	1
	42985	0	0	0	0	0	0	0
287	42355	1	0	0	0	0	1	1
	42985	53	0	0	0	1	53	53
315	42355	0	0	0	0	0	0	0
	42985	0	0	0	0	0	0	0
388	42383	6	0	0	0	0	6	6
	42562	69	0	0	0	1	69	69
287	42528	62	12	0	0	1	74	86
	42987	86	43	40	13	3	182	344
380	42526	0	0	0	0	0	0	0
	42747	16	11	0	0	0	27	38
419	42526	6	0	0	0	0	6	6
	42747	17	4	0	0	0	21	25
287	42145	79	76	56	37	4	248	547
	42743	63	48	44	36	4	191	435
414	42738	1	0	0	0	0	1	1
	42985	0	0	0	0	0	0	0
	42355	1	0	0	0	0	1	1
287	42747	40	35	16	0	2	91	158
	42526	43	10	6	0	1	59	81

DISCUSSION

Accuracy and objectivity in the counting of target cells for the MCI were achieved. Also, the tedious counting or scoring was eliminated through electronic image analysis. Although variability was less when tests were counted by the same person, satisfactory agreement in counts for the same tray was obtained by different operators. At higher target cell numbers per

well, errors were introduced by crowding and clustering of cells which the scanner could not separate. This was not reflected in the relative error, since it was repeated each time the well was counted. At the other end of the scale, variability increased considerably. This can be seen in the high relative errors as numbers dropped below 25. Approximately 50–300 target cells appeared to be best suited for counting with the Quantimet 720. Because of reduction in the number of target cells during the test, an attempt was made to plate from 100 to 300 cells per well.

Placing of tests and controls on the plate must be carefully considered (because of the existence of position effects). Interpretations of lower target cell numbers as positive tests in outside wells of the plates, in comparison to more central regions of the plates, give erroneous results. In our tests, we reduced the possibility of false positives from these effects by dividing the plate into quadrants with controls on the sides of the plate. Target cell numbers had to be reduced below the region of poorest growth giving the most conservative estimate of target cell reduction. When feeder effects from added lymphocytes were noted, percent target cell reduction was related to the results of wells with the lowest level of lymphocytes. Thus the control level of no target cell reduction was taken from wells with either the fewest or no lymphocytes and which showed the maximum target cell survival. Using such controls, we could detect that normal individuals possessed lymphocytes which often reacted with target cells. The variability in effect with lymphocytes from normal individuals indicated that this group could not be used as negative controls.

Because the microassay is carried out in as little as 10 μl of medium, depletion of nutrients has been mentioned as a possible cause for reduction of target cell survival. Increasing the volume to 15 μl and to flooded plates made little difference except perhaps that it caused a slight increase in sensitivity. Differences in sensitization were detected after 1-day incubation, although increasing the duration of the test also increased target cell reduction. A leveling of cell-mediated effects was noticed after the 3d day. To avoid in vitro sensitization (*12*, *13*) and the increase in variability with longer intervals of incubation, the test was terminated at 2 days.

If one considers the many variables involved in cell-mediated tests, it is not surprising that exact reproducibility is not achieved in testing patients on different occasions. The degree of sensitization against antigens on surfaces of target cells by patients must vary each day as does the representation of the antigens on target cells from different passages. Technical difficulties of isolating lymphocytes with different ratios of contaminating granulocytes contribute to slight variations as do small uncontrollable differences in the numbers of effectors and target cells used. Because these factors are difficult to control, tests should be repeated as often as possible. Although we believe that reproducibility needs to be improved, valuable information on sensitization in patients and specificities of reaction can be obtained with techniques presently available. The solution of the problem of counting target cells through the development of a rapid and efficient method of image analysis allows transfer of efforts toward the more central problems in cancer.

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Use of an In Vitro Microcytotoxicity Test to Assess Human Tumor-Specific Cell-Mediated Immunity and Its Serum-Mediated Abrogation [1, 2]

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SUMMARY—We used the microcytotoxicity test to study cell-mediated immunity and its serum-mediated abrogation in 69 patients with intracranial tumors and 6 with malignant melanoma. Important to our success was the use of improved tissue-culture procedures and modifications to minimize the nonspecific reactivity of control lymphocytes. Best results were obtained with target cells derived from homogeneous (cloned if necessary) cell cultures in an early passage. Recent studies used 5-^{125}iodo-2′-deoxyuridine-labeled target cells.—Natl Cancer Inst Monogr 37: 85–92, 1973.

THE INHIBITORY activity of the tumor patient's lymphocytes against his own tumor cells, or those of a patient with a similar neoplasm, has been studied in vitro by a variety of methods (*1*). The same assays have been used to assess the ability of patient's serum to abrogate the lymphocyte-mediated response and provide a possible in vitro correlate of immunologic enhancement. We have used a modification of the Hellström microcytotoxicity test (*2*) to study 75 patients—69 with primary intracranial tumors and 6 with malignant melanoma. In principle, this test is quite simple and logical: Fewer tumor cells would survive and/or undergo mitosis in the presence of immune lymphocytes from tumor patients than in the presence of lymphocytes from normal controls. Several laboratories, however, could not assess tumor-specific cell-mediated immunity (CMI) in this way. Most of their difficulties centered about cultivation and selection of appropriate target cells or the presence of unacceptably high levels of antitumor cell activity in the control lymphocytes. This paper describes the means we have used to minimize these problems.

MATERIALS AND METHODS

Tissue culture.—Medium (TCM) was Eagle's minimal essential medium (MEM) with twice-concentrated vitamins, glutamine, and arginine, supplemented with Eagle's "nonessential" amino acids, biotin 1 mg/liter, inositol 16 mg/liter, choline chloride 13 mg/liter, $FeSO_4 \cdot 8H_2O$ 0.8 mg/liter, $ZnSO_4 \cdot 7H_2O$ 0.8 mg/liter, medium NCTC 135 5%, penicillin 100 U/ml, and kanamycin 50 μg/ml. Biopsy specimens of tumor, normal brain, or skin were carefully dissected to remove as much of the vascular and connective tissue elements as possible and then cut into pieces 1–2 mm across. The tissue was washed free of blood in phosphate-buffered saline (PBS), and some was explanted into plastic culture flasks (Falcon #3012). The explants were kept

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dry for 30–60 minutes to facilitate adherence; then each piece was fed with a single drop of TCM containing 20% fetal bovine serum (FBS) (Grand Island Biological Co.) and incubated 1 or 2 days in a humidified atmosphere of 5% CO_2 in air. The entire flask was then fed with 4 ml of TCM-FBS. The nonexplanted portion of the tissue mincate was incubated with slow stirring at room temperature for 5–20 minutes in a 0.125% solution of trypsin (General Biochemicals, #70730) in Puck's Saline A. Debris was removed by 1-minute sedimentation in a 50-ml conical centrifuge tube, and the cell suspension was transferred to a second tube and spun at 150 × *g* for 10 minutes at 4° C. The cells were resuspended in 1–10 ml of TCM containing 2% FBS and counted; 10^5–10^6 cells were then seeded into several culture flasks containing 4 ml TCM with 20% FBS. In all cultures, 75% of the medium was changed twice weekly. All cultures were examined every 2–3 weeks for mycoplasma contamination by aerobic and anaerobic culture on agar (Grand Island Biological Co., #804AP). Any contaminated culture was discarded.

By phase-contrast microscopy, a flask was selected that contained a homogeneous population of the appropriate cells. If no such flask was found, cloning or treatment with agamma FBS was performed to obtain a morphologically uniform culture. Cells from the second to fourth passage of the selected culture were grown in several 75-cm² plastic flasks (Falcon #3024), frozen in portions of 10^5–10^6 cells in TCM with 20% FBS and 10% dimethyl sulfoxide, and stored in liquid nitrogen. When target cells were needed for the microcytotoxicity test, a vial was thawed and the cells were grown to confluence. The monolayer was dispersed with 0.025% trypsin, and the cells were seeded at the appropriate concentration in Falcon Microtest II plates (#3040).

Cloning of tumor cells.—We used two methods. By the first method, cells were seeded in 100-mm plastic culture dishes (Falcon #3003) at 100, 500, 1,000, and 5,000 cells per dish and allowed to adhere overnight. Appropriate single cells were isolated within a 5-mm porcelain ring, allowed to proliferate, and then transferred to a culture flask (Falcon #3012) by trypsinization. By the second method, 100, 500, and 1,000 cells were seeded into 35-mm plastic culture dishes (Falcon #3001) containing a finely fractured glass coverslip. The next day coverslip pieces bearing a single cell were transferred to individual wells of a Microtest II plate. The cells were grown to confluence and transferred to a culture flask by trypsinization. TCM with 20% FBS was used in both procedures.

Selective plating of tumor cells in agamma FBS.—Tumor cell cultures contaminated with fibroblastic cells were trypsinized as usual. A total of 1×10^5 viable cells was seeded in each of five 25-cm² culture flasks containing 4 ml TCM with 20% γ-globulin-free FBS (Grand Island Biological Co., #651) and incubated 12 hours at 37° C. Cultures were washed once with PBS, refed with TCM containing 20% FBS, and then handled as usual. If a few fibroblasts persisted, cultures were kept on TCM-FBS for 12 hours, washed, refed with TCM-agamma FBS for 12 hours, again washed, and then maintained on TCM-FBS as usual.

Microcytotoxicity assay.—Tumor cells and appropriate normal cells were seeded in Microtest II plates at a concentration adjusted to give 50–100 adherent cells per well by the following day. For 64 of our 75 patients, we had autologous normal cells as well as tumor cells. The plates were washed with warm TCM containing 0.015M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (Sigma Chemical Co.). Various dilutions (0.1 ml) of the sera or fractions to be assayed for blocking activity were added for 45 minutes at 37° C. Controls were given similar dilutions of human AB+ serum. Sera were aspirated, and 1×10^5 lymphocytes were added to each well. The lymphocytes were isolated as described in (*3*) and suspended in 0.2 ml TCM-HEPES containing 10% human AB+ serum (different from control serum). Control lymphocytes were from healthy males and nulliparous females, none of whom had ever been transfused. All control and experimental lymphocytes were from donors ABO–compatible with the donors of all target cells. Plates were incubated 18 hours on a rocker at 37° C and an additional 24 hours without the rocker; then they were washed with PBS, fixed with methanol, and stained with Giemsa. The total number of target cells in each well was

counted. All tests were done in quadruplicate to sextuplicate.

The cell-mediated response (CMR) was expressed as percent inhibition calculated as shown in text-figure 1. Blocking activity was expressed as the percent reduction in CMR in the presence of experimental serum as compared to the CMR in the presence of control serum.

$$\% \text{ Inhibition} = \left\{ 1.0 - \frac{\dfrac{\text{overage \# tumor cells (experimentol)}}{\text{average \# tumor cells (controls)}}}{\dfrac{\text{overage \# normol cells (experimental)}}{\text{average \# normol cells (controls)}}} \right\} \times 100$$

TEXT-FIGURE 1.—Formula for expression of microcytotoxicity data.

Modification of the microcytotoxicity test with 5-^{125}iodo-2'-deoxyuridine (^{125}IUDR)-labeled target cells.—Microtest II plates were seeded with 500 or 1,000 cells per well. The next day the medium was aspirated, and 0.1 ml of TCM containing 20% FBS, ^{125}IUDR 10 μCi/ml (New England Nuclear Corp., #NEX–072), and 10^{-6}M fluorodeoxyuridine (Hoffmann-LaRoche) was added to each well. Plates were incubated 24 hours at 37° C, after which the assay was performed. Plates were not stained but rather were washed twice with PBS and thrice with methanol, air-dried, and then sprayed with Rezifilm (E. R. Squibb & Sons, Inc.). The bottom of each well was punched out with a one-fourth inch bolt and counted in a Baird Atomic gamma spectrometer. Results were calculated by the formula in text-figure 1, substituting average counts per well minus background for average number of cells per well.

RESULTS AND DISCUSSION

Tables 1 through 3 summarize our results. Of the 75 patients studied, 67 had CMI against their own tumor cells statistically significant at the 5% level or better; 57 of 67 had CMI against allogeneic tumor cells of the same type as their own, and 46 of 65 had serum blocking activity. Presented elsewhere (*3, 4*) is evidence for cross-reactivity between the CMI to well-differentiated (fibrillary astrocytomas) and anaplastic (glioblastoma multiforme) tumors of the same cell type and between glioblastoma and melanoma but not between glioblastomas or astrocytomas and sarcomas or carcinomas. Moreover, blocking activity has been recovered in the pseudoglobulin fraction and in the IgG eluates from diethylaminoethyl-cellulose (*4*).

Improved Tissue Culture Conditions: Selection of Tissue Culture Medium

Optimal maintenance of the cultured cells is central to the microcytotoxicity test, both in providing appropriate target cells and in minimizing "spontaneous" target cell loss during the test. Table 4 lists in descending order of effec-

TABLE 1.—*Cell-mediated immunity to autologous tumor cells*

Tumor type*	Distribution of patients by percent inhibition†			
	25–40	40–55	55–80	Statistically insignificant‡
Glioblastoma multiforme	2	7	2	3
Malignant astrocytoma	2	4	1	1
Fibrillary astrocytoma	2	8	1	2
Ependymoblastoma		2		1
Ependymoma		2		
Oligodendroglioma	1	1		
Ganglioglioma		2		
Mixed glioma	2	3		
Meningioma	4	12	3	1
Melanoma	2	3	1	

*Tumors were classified according to the *Systemized Nomenclature of Pathology*, established in 1965 by the American College of Pathologists.

†Calculated by formula shown in text-figure 1. When normal target cells autologous to the tumor cells were unavailable, CMI was calculated as the percent reduction in tumor cells by experimental lymphocytes relative to the number of target cells remaining after exposure to control lymphocytes. In no such instance did normal lymphocytes produce >20% inhibition relative to controls treated with medium alone.

‡The probability was >5% that the average numher of cells remaining after treatment with patient's lymphocytes was equal to that remaining after treatment with control lymphocytes.

TABLE 2.—*Cell-mediated immunity to allogeneic tumor cells of the same histologic type as that of the patient*

Tumor type*	Distribution of patients by percent inhibition†			
	25–40	40–55	55–80	Statistically insignificant‡
Glioblastoma multiforme	2	6	2	4
Malignant astrocytoma	2	3		2
Fibrillary astrocytoma	3	6	1	2
Ependymoblastoma		1	1	1
Mixed glioma	2	2	1	1
Meningioma	5	13	1	1
Melanoma	2	3	1	

*Tumors were classified according to the *Systemized Nomenclature of Pathology*, established in 1965 by the American College of Pathologists.

†Calculated by formula shown in text-figure 1. When normal target cells autologous to the tumor cells were unavailable, CMI was calculated as the percent reduction in tumor cells by experimental lymphocytes relative to the number of target cells remaining after exposure to control lymphocytes. In no such instance did normal lymphocytes produce >20% inhibition relative to controls treated with medium alone, nor did patient's lymphocytes produce >20% inhibition of allogeneic fibroblasts or normal glial cells.

‡The probability was >5% that the average number of cells remaining after treatment with patient's lymphocytes was equal to that remaining after treatment with control lymphocytes.

TABLE 3.—*Blocking factor activity against autologous* cell-mediated immunity to autologous tumor cells*

Tumor type†	Distribution of patients by percent blocking activity‡				
	25–40	40–55	55–70	70–100	Statistically insignificant§
Glioblastoma multiforme	1	1	3	4	1
Malignant astrocytoma		1	3	1	1
Fibrillary astrocytoma	2	2	2	1	4
Ependymoblastoma		1		1	
Ependymoma		1			1
Oligodendroglioma			1		1
Ganglioglioma			1		1
Mixed glioma	1		1	1	2
Meningioma	6	3	1	2	7
Melanoma			2	3	1

*Table does not consider results from patients who own lymphocytes were not inhibitory but whose sera did have blocking activity when tested with reactive lymphocytes from another patient with a similar tumor.

†Tumors were classified according to the *Systemized Nomenclature of Pathology*, established in 1965 by the American College of Pathologists.

‡Calculated by comparing CMI (text-fig. 1) in presence of patient's serum to CMI in presence of control serum.

§The probability was >5% that the CMI in the presence of patient's serum was equal to that in the presence of control serum.

tiveness several commonly used media, tested simultaneously on the 4 cell types commonly grown in our laboratory. The ability to support cloning was assessed on 1 meningioma, 1 melanoma, and 2 glioma cultures with the cloning ring method described above.

TCM was formulated empirically by combining several features of Eagle's MEM, Puck's N16, and Ham's F12. It was clearly superior to any of the other media tested. Of the commercially available media, little difference was observed between MEM, N16, and F12. We were concerned that cells grown in TCM would offer an advantage to TCM in a comparison of the various media. All cells used in the test had, therefore, been maintained through 2 passages in medium NCTC 135 containing 20% FBS. It is interesting that the 2 media judged least efficient were the ones most commonly used in other laboratories for the microcytotoxicity test. RPMI 1640 was originally devised to support leukemoid cells in suspension culture. Its high phosphate and low divalent cation concentration make it a poor choice for culture of adherent

TABLE 4.—*Comparison of various media's ability to support plating, growth, and cloning of cultured cells*

Medium*	Cell type											
	Fibroblasts			Glioma cells			Meningioma cells			Melanoma cells		
	PE† (%)	Growth‡ (days)	Cloning	PE (%)	Growth (days)	Cloning	PE (%)	Growth (days)	Cloning	PE (%)	Growth (days)	Cloning
TCM	78	4	NT§	74	3	+	81	3	+	51	4	+
Eagle's MEM with "nonessential" amino acids	58	6	NT	51	5	−	72	4	−	39	5	NT
Puck's N16	63	5	NT	46	5	−	68	6	−	29	6	NT
Ham's F12	47	6	NT	49	6	+	67	7	−	36	7	NT
Eagle's MEM	34	7	NT	36	6	−	36	6	−	19	7	NT
Ham's F10	39	8	NT	29	8	−	28	8	−	18	8	NT
Waymouth's MB 752/1	32	8	NT	17	10	−	32	9	−	16	9	NT
RPMI 1640	19	9	NT	14	13	−	18	8	−	11	12	NT

*All media prepared in our laboratory according to the formulae in the Grand Island Biological Co. catalog and supplemented with 20% FBS, penicillin 100 U/ml, and kanamycin 50 μg/ml.

†Plating efficiency (PE) determined by seeding 300 viable cells into each of 10 wells of a Microtest II plate and counting total adherent cells per well after 12 hours at 37° C.

‡Five 25-cm² flasks were each seeded with an inoculum sufficient to give 5×10^4 adherent cells with each of the media (calculated according to plating efficiency). Cells were fed every 3 days, and the time when a confluent monolayer had formed was recorded.

§NT = not tested.

cells. Waymouth's medium is very rich but does contain some amino acids at concentrations found inhibitory to growth of certain cells (*5*).

Advantage of the Use of Tumor Cells From Homogeneous Cultures

Contamination of tumor cell cultures by normal fibroblasts is a common problem. These fibroblasts often proliferate faster than the tumor cells and thus may overgrow the whole culture. Moreover, use of tumor cell cultures composed even in part of fibroblasts to provide tumor target cells for the microcytotoxicity test would diminish the percentage of "tumor cells" sensitive to the cytotoxic action of tumor-specific immune lymphocytes.

We have used 3 methods to obtain homogeneous tumor cell cultures. However, there is generally no foolproof method to distinguish, in living cultures, human tumor cells from normal fibroblasts or glial cells. Indeed, we have seen skin fibroblasts assume a variety of shapes and sizes, including the large, flat, leaflike appearance characteristic of some epithelioid cells. Occasionally, one can use markers like the pigment in a melanoma cell, but usually one must rely on judgment in selecting the tumor cell from a mixed population. When there is doubt, the culture should not be used. We have cloned 27 of 41 cultures with the methods described above. The agamma FBS technique has been successful in 13 of 19 attempts; 5 successes occurred where cloning had failed. Why agamma FBS can be used to select neoplastic over normal cells is unclear but probably rests, at least in part, on the use of alcohol precipitation to remove the γ-globulin. Such treatment precipitates not only γ-globulins but also α-globulins. Normal cells may be more dependent than neoplastic cells on undefined growth factors in the α-globulin fractions (*6*).

Table 5 shows the effect of homogeneous tumor cell populations on the microcytotoxicity test. Tumor cell cultures before and after cloning or agamma FBS treatment were simultaneously tested against autologous lymphocytes. The percent inhibition was always greater when homogeneous tumor cells were used. To insure that the increased effect was due only to the elimination of fibroblasts and not to the use of a cloned population in general, 2 cultures (T12 and T46) morphologically homogeneous without cloning were cloned anyway and included in the study. Here cloning had no effect on the microcytotoxicity test results.

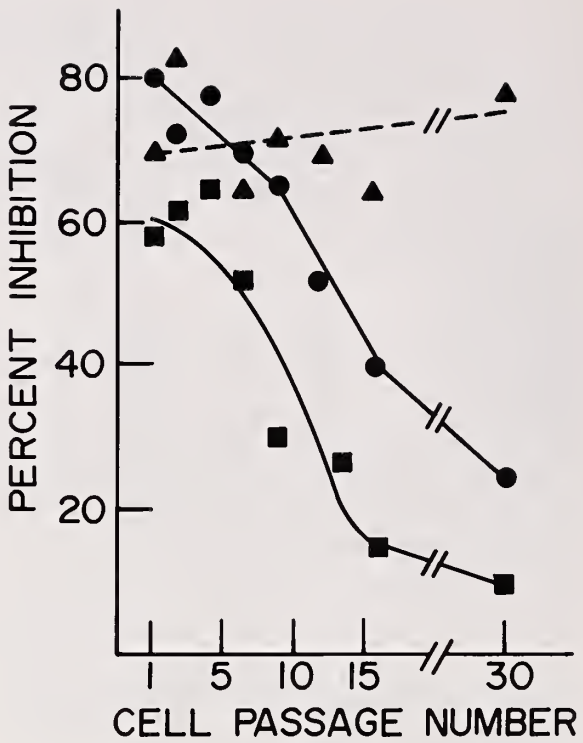
Advantage of the Use of Early Passage Target Cells

The optimal stage at which cell cultures

TABLE 5.—*Effect of homogeneous target cell populations on the microcytotoxicity test*

Target cell	Treatment	Percent inhibition by autologous lymphocytes
T42	None	40
	Cloned	76
T56	None	22
	Cloned	51
T57	None	38
	Agamma FBS	69
T63	None	29
	Cloned	48
T12	None*	46
	Cloned	41
T46	None*	59
	Cloned	64

*Culture morphologically homogeneous before cloning.



TEXT-FIGURE 2.—Effect of prolonged tissue culture on tumor cell sensitivity to cellular immune inhibition. Three cell lines were frozen-stored at various passages, and cells from the different passages were compared as target cells in a single microcytotoxicity test. Autologous lymphocytes from a single preparation were used. The 3 cell lines had all been cloned, had grown well in culture throughout the study, and were mycoplasma-free in 3 serial examinations. *Triangles* represent T52, a fibrillary astrocytoma; *squares*, T47, a melanoma; and *circles*, T63, a fibrillary astrocytoma.

should be used as target cells in the microcytotoxicity assay has not been determined. There are 3 alternatives:

1) Primary cultures have a minimum of potential tissue culture-induced alterations. Such cultures seldom would be homogeneous, however, and might also have patients' serum factors adsorbed to their surface. Such factors might complicate test interpretation. If primary cultures were used, one would have to perform the assay immediately after surgery, when the operative stress, drugs, and transfusions are most likely to influence the patient's immune response.

2) Long-term lines are the most convenient cell sources. They are readily maintained in culture, often easily cloned, and if in culture for 6 months or more, usually free of non-neoplastic cells. Such cells, on the other hand, are most likely to acquire occult viral or mycoplasma infection and, most importantly, to undergo some alteration in their surface antigens. Text-figure 2 shows the results of an experiment conducted to see if prolonged culturing does alter the sensitivity of tumor cells to immune lymphocytes.

T52's responsiveness remained stable throughout the 30 passages. T47 and T63 did not change for the first several passages but then underwent a slowly progressive diminution in sensitivity to cellular immune inhibition. The cause of this decreased sensitivity—be it diminished or altered antigenicity or simply a selection of less "fragile" cells—does not alter the fact that prolonged culture did reduce the usefulness of these 2 cell lines as target cells in the microcytotoxicity test.

3) The third alternative, and the one that we have chosen, is to use cells in an early tissue-culture passage. It is unlikely that patients' serum factors would remain adsorbed to the cells after a few passages by trypsinization. Since we routinely use only homogeneous cultures, the potential advantage of long-term lines (that they are probably homogeneous tumor cell populations) is at least partially obviated. In the microcytotoxicity assay, we always use cells from the second to fifth passage and thereby hope to

minimize and standardize any tissue culture-induced target cell alterations. Frozen samples of the same cells are used each time the test is repeated, which facilitates comparison of test results obtained on different days.

Efforts to Minimize the Background Cytotoxicity Induced by Control Lymphocytes

Recently (*3*) we showed that control lymphocytes may induce greater inhibition of tumor target cells than do patients' lymphocytes. We referred to the control lymphocyte activity as "nonspecific," however, since it was also directed against normal target cells autologous to the tumor cells. The patients' lymphocytes, on the other hand, had little or no activity against normal target cells. This nonspecific control activity has been a major problem in the interpretation of microcytotoxicity results and emphasizes the need for normal target cells autologous to the tumor cells. We did present (*3*) several modifications of the assay that minimized the nonspecific control activity, however, and facilitated analysis of experiments in which no normal target cells were available. These measures included reduction in the number of lymphocytes per well, use of human AB+ serum instead of FBS in the assay, ABO matching of lymphocyte and target cell donors, and reduction of the final incubation time from 3 days to 40 hours. Recent studies have confirmed the efficacy of these measures but have not clarified why they worked.

Since ABO matching had helped reduce control reactivity, we examined the effects of HL-A incompatibility. Two different pairs of tumor cells and autologous fibroblasts were exposed to serial dilutions of autologous lymphocytes and lymphocytes from unrelated donors with varying degrees of phenotypic HL-A identity with the target cell donors. No lymphocyte donor had ever been pregnant, transfused, or otherwise immunized against the target cell donor's tissues. Table 6 suggests that, unlike with ABO, phenotypic compatibility at HL-A had little, if any, effect on the inhibition of neoplastic or normal cells by lymphocytes from nonpresensitized individuals. Before we can discard HL-A or

TABLE 6.—*Effect on nonspecific target cell inhibition of phenotypic HL-A compatibility between target cell and lymphocyte donors*

Target cells (HL-A type)*	Lymphocyte donor		Percent inhibition† at various lymphocyte dilutions				
	HL-A	ABO	10^5	3×10^5	6×10^5	10^6	3×10^6
Glioblastoma (1,13,9,W5)	Autologous	A	68	77	86	89	88
	1,13,9,W5	O	18	22	31	60	92
	1,8,10,W5	O	19	38	48	39	59
	1,8,10,5	O	22	15	29	56	74
	3,8,W15,W19	A	6	8	18	30	49
	3,8,11,W22	O	19	26	43	61	84
Fibroblasts (1,13,9,W5)	Autologous	A	−2	−9	4	21	18
	1,13,9,W5	O	19	36	49	61	78
	1,8,10,W5	O	13	30	51	78	76
	1,8,10,5	O	26	46	70	73	86
	3,8,W15,W19	A	−3	10	29	46	50
	3,8,11,W22	O	14	29	41	58	69
Meningioma (1,8,3,7)	Autologous	A	53	69	82	91	86
	1,8,3,7	O	21	48	59	60	71
	1,8,10,W5	O	26	29	38	57	68
	1,13,9,W5	O	19	27	46	71	64
	2,W16,10,5	O	24	36	49	63	72
	1,W15,2,12	B	12	25	36	51	65
	2,W15,11,W27	A	3	9	21	46	51
Fibroblasts (1,8,3,7)	Autologous	A	−7	−3	19	26	30
	1,8,3,7	O	18	56	68	69	76
	1,8,10,W5	O	21	30	46	61	70
	1,13,9,W5	O	24	46	52	68	76
	2,W16,10,5	O	13	27	48	71	73
	1,W15,2,12	B	21	38	54	56	68
	2,W15,11,W27	A	6	14	27	53	56

*HL-A type of target cells determined by standard typing of patients' lymphocytes.
†Expressed as percent reduction compared to wells treated with medium alone.

HL-A-linked effects, however, we must perform similar tests within the family of the target cell donor, where we can compare the effects of lymphocytes from individuals genotypically HL-A identical to the target cell donor to the effects of lymphocytes from individuals differing from the donor by one or both haplotypes. Serologic HL-A identity does not imply that all 4 antigens are truly identical, nor does it provide any information on possible ancillary genetic loci, linked to HL-A, but responsible for delayed hypersensitivity and mixed leukocyte reactivity (*7*). Indeed, these ancillary loci may be of central importance to the cytotoxic potential of immune and nonimmune lymphocytes both in vivo and in vitro.

Use of ^{125}IUDR-Labeled Target Cells

Visual counting of cells in the standard microcytotoxicity test is a laborious, somewhat subjective process. Use of labeled target cells obviates these difficulties. The advantages of ^{125}IUDR over ^{3}H-thymidine and ^{51}chromium were discussed by Cohen et al. (*8*). We have used the ^{125}IUDR technique to study 14 patients and have obtained results comparable to those shown in tables 1 through 3. We found no increase in control reactivity over that in the standard procedure but noted a slight decrease in the calculated percent inhibitions. This decrease may in part be due to the fact that, since ^{125}IUDR-labeled cells do not divide, that component of immune inhibition related to proliferation inhibition was not included. We now routinely use labeled target cells in our microcytotoxicity studies.

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Cytotoxicity of Human Peripheral Lymphocytes for Glioma, Osteosarcoma, and Glia Cell Lines [1, 2]

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SUMMARY—Three series of experiments were conducted to examine the cytotoxic effects of peripheral blood lymphocytes from glioma, subarachnoidal hemorrhage (SAH), and other non-brain-tumor patients against glioma, normal glia, and osteosarcoma cell lines. The pattern of cytotoxicity appeared to reflect target cell sensitivity rather than tumor specificity. When cytotoxic to glioma cells, the lymphocytes from glioma patients were also cytotoxic to the other target cells. A tumor-specific effect was apparent against one of the glioma cell lines when compared to the effect on the normal glia cells. Lymphocytes from the nontumor patients often produced cytotoxic effect against the target cells that was most pronounced with patients with SAH. Often presensitization by transfusion or pregnancy could be ruled out as causing this cytotoxicity. The importance of the use of several target cell lines and varied lymphocyte concentrations is emphasized.—Natl Cancer Inst Monogr 37: 93–102, 1973.

THIS SERIES of investigation was started to search for tumor-specific lymphocyte cytotoxicity in glioma patients. The best test condition for such studies is the in vitro confrontation of tumor cells and lymphocytes from the same patient. Glioma-specific reactivities were reported previously in autologous tests (*1, 2*). However, if this is the only possible condition, such studies would be limited when the immune reactivity of several patients is to be followed. In fact, tumor-specific reactivity of peripheral lymphocytes from tumor patients was demonstrated against allogeneic target cells in several studies, in which established cell lines were also used (*3–7*).

One puzzling feature of these studies is the cross-reactivity between patients with tumors in the same organ. In view of the results with animal tumors showing cross-reactivity between tumors induced by the same virus even if they arise in different tissues, contrasted to the individually distinct antigens of chemically induced tumors, the question of the nature of the human tumor antigens is not resolved. The pattern obtained in human tumors may suggest the expression of organ-specific antigens on tumor cells.

Since several established glioma and glia lines are available (*8*), we could include in our tests the normal counterpart of the malignant cell.

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[6] We acknowledge the expert technical assistance of Karin Kvarnung.

Previous reports indicating autoimmune phenomena against glial elements (*9*) also motivated the plan of this study. The osteosarcoma line 2T was included as a control; this is one of the cell lines selected for comparative studies carried out in collaboration between different laboratories (*10*).

MATERIALS AND METHODS

Target cells.—Established glioma cell lines 118 MG, 138 MG, and 178 MG, 2 glia lines 587 CG and 622 CG, and 1 osteosarcoma line 2T were obtained from Jan Pontén, Department of Pathology, Uppsala, Sweden (*8*). Another glia line 336 CG was established in our laboratory by F. Vánky. The cells were suspended from the monolayer by treatment with 0.25% trypsin. About 300 cells were seeded in each well of the Falcon 3034 microplate in 20 μl minimal essential medium (MEM) containing 10% fetal calf serum (FCS) (*11*).

Effector cells.—Heparinized blood (40 ml) was sedimented in plasmagel for 30–60 minutes at room temperature. The leukocyte-rich plasma was collected and incubated for 45 minutes at 37° C in a nylon fiber column. The column was eluted with MEM containing 1% FCS. The cells were collected by centrifugation. Erythrocytes were removed by Tris ammonium chloride treatment. After 3 washings with MEM containing 1% FCS, the cells were resuspended in MEM containing 10% FCS. Viability was >95%, and the population was usually about >95% lymphocytes as judged by stained smear preparations.

Cytotoxic tests.—Individual lymphocyte suspensions (2–4–6 at each occasion) were tested in 5 double-dilution series starting from numbers which gave approximately 200:1 lymphocyte-target cell ratio. The actual number was calculated on the basis of target cells counted at the bottom of the well. For each sample, 6 wells were used. For adequate humidity, the wells were surrounded with medium at the edge of the microplate. At each occasion, lymphocytes from at least one control were included. After 48 hours' incubation in CO_2, the plates were rinsed with BSS, and the remaining cells were fixed with methanol and stained with May-Grünwald and Giemsa. The arithmetic mean of the cel. numbers in 6 samples was calculated.

Patients.—Three series of experiments were performed. Series I included 12 patients with glioma of different degrees of differentiation (astrocytoma grade I, II, III, IV), 3 patients with subarachnoidal hemorrhage (SAH), and 14 patients with trigeminal neuralgia or obsessive neurosis. Series II included 5 patients with glioma and 5 controls. Some tumor patients could be tested before surgery.

The results were summarized after the first series. When series III was started, a new batch of cell lines was obtained from Dr. Pontén including the previously used ones, a third glioma cell line, and a new glia line. This series included 16 patients with glioma, 6 with SAH and 7 controls. The time intervals for the series were: I, Oct. 1971–Jan. 1972; II, Jan.–March 1972; and III, March–May 1972.

Evaluation of results.—The cytotoxic effect of each lymphocyte preparation was evaluated in relation to the target cells without the addition of lymphocytes, i.e., the medium control. Lymphocyte-containing wells often showed higher target cell numbers. When this effect was considered, cytotoxicity by high lymphocyte numbers was manifested by the decrease or lack of stimulation and a low degree of actual decrease of the cell numbers compared to those in the medium control. The stimulatory effect was not uniform even in the same experiment with different lymphocyte suspensions. It seems therefore important to titrate the lymphocytes rather than to use only one cell dose. In the evaluation, we considered a test positive if the target cell reduction with the ratio 200:1 was >20% (series I) or 30% (series III) or if we could see a clear titration effect.

RESULTS

Four experiments with 2–4 patients on 4 or 5 target cells are detailed in table 1. To exemplify the results, the actual counted number/wells with the standard error is indicated for 2 target cells in experiment I. The remaining results are given as percentage of target cell reduction at the different lymphocyte-target cell ratios. In experiment I, cytotoxicity was strong with most

TABLE 1.—*Cytotoxic assays on 5 target cells. The consecutive numbers give the results for 200, 100, 50, 25:1 effector-target cell ratios*

Experiment	Patient No.	Sex	Age (yr)	Diagnosis	Target cells/well									
					118 MG					138 MG				
I 1/18/72	35	♂	32	Epilepsy, metastatic	15.3±2.1	34.0±4.1	88.7±14.6	95.3± 6.6	(74.8±14.9)*	50.2±5.5	63.2±8.1	64.2±5.0	63.8±12.7	(66.2±6.8)*
	47	♂	45	Adenocarcinoma	9.2±2.4	15.2±2.5	28.3± 8.2	32.5± 6.2	(46.6± 6.4)	29.3±6.3	30.2±6.8	34.0±8.0	39.8± 7.0	(45.0±9.3)
	48	♂	40	Astrocytoma II	6.3±2.3	17.0±1.2	45.3±10.1	70.8±16.6	(74.8±14.9)	18.8±5.7	14.5±3.4	28.7±7.1	41.2±10.5	(46.0±1.2)
	49	♂	67	Hydrocephalus	11.7±2.8	7.0±1.5	19.3± 3.1	24.8± 4.7	(47.7± 7.1)	22.8±2.6	37.3±5.5	45.5±7.1	50.3± 5.3	(66.2±6.8)

Experiment	Patient No.	Sex	Age (yr)	Diagnosis	Percentage of cell reduction															
					Glioma								Osteosarcoma				Glia							
					118 MG				138 MG				2T				587 CG				836 CG			
I 1/18/72	35	♂	32	Epilepsy, metastatic	80	55	−19	−28	24	4	3	4	50	6	−2	−4	6	3	2	3				
	47	♂	45	Adenocarcinoma	81	67	40	30	35	33	24	12	66	41	24	5	17	15	0	−1				
	48	♂	40	Astrocytoma II	92	77	39	5	60	69	37	10	54	12	−4	−19	−10	−11	−14	−5				
	49	♂	67	Hydrocephalus	76	85	60	48	66	44	31	24	21	12	−19	−22	6	−4	−10	−6				
II 2/15/72	55	♂	41	Obsessive neurosis	−23	−24	−5	−10	−6	−17	12	−20	5	−4	−2	−5	12	5	−2	−6	−4	−4	−3	−14
	56	♂	57	Astrocytoma I	68	62	38	16	72	55	46	26	53	38	13	−4	6	6	−3	−2	−19	−10	−10	−12
	57	♀	63	Astrocytoma II	−4	3	−2	3	32	33	16	16	−8	−3	−10	−8	−1	−4	6	2	−5	0	0	0
III 2/22/72	58	♀	70	Astrocytoma IV	76	65	22	15	71	61	47	9	−1	18	10	10	46	32	22	23	10	0	5	6
	59	♀	44	Obsessive neurosis	−24	−34	−8	3	12	−10	2	18	14	11	6	10	29	1	3	4	7	18	12	5
IV† 5/3/72	72	♂	43	Astrocytoma III	9	−14	−38		24	−6	−9	−17	45	24	6	−2†	14	6	0	0				
	75	♀	31	Obsessive neurosis					38	24	29	20	22	12	18	37	34	2	−13	4				
	67	♀	58	SAH					83	41	20	8	56	38	23	5	29	26	−4	−16				
	45	♀	63	Astrocytoma III	52	22	6	10	−2	−6	3	−3	32	18	9	−12	17	19	13	−8				

*The numbers in parenthesis are values for the medium control.
†In this test, glia line 622 was used.

combinations except with the glia cell targets. The osteosarcoma cells were affected by all lymphocytes.

We often observed that added lymphocytes stimulate the target cells. Therefore, on the basis of the clear titration effect, the lymphocytes of patient 49 could also be considered toxic to 2T cells even though they gave only 21% reduction at the 200:1 ratio.

In experiment II, only patient 57 showed a clear titration effect. However, this effect was not glioma specific, since it was also seen against the osteosarcoma. It might have had a specific component, since the glioma lines were affected at a lower ratio—50:1.

Experiment III revealed a tumor-specific reactivity, since only the glioma cells were damaged in a clear, dose-dependent way by the lymphocytes of the glioma patient. A slight effect on glia cell 587 could also be noted.

Some dose-dependent cytotoxicities were seen in certain combinations in experiment IV. The pattern, however, was not clearly tumor or organ specific. The lymphocytes of patient 72 were positive against all 4 cell lines, with a low effect against the glia cell. The lymphocytes of the other tumor patient—45—were positive against one of the glioma lines and the osteosarcoma line. While lymphocytes of control patient 75 had no, or possibly a slight, effect on the 622 line, the lymphocytes of the patient with SAH were strongly positive against all 3 cell lines tested.

The results of these 4 tests suggest that the glia lines may be less sensitive to the cytotoxic effect of lymphocytes and, furthermore, that lymphocytes from control patients whose general condition was not affected are less often cytotoxic.

The first summarizing evaluation was made after results were obtained from 12 glioma patients (series I). Table 2 gives for each lympho-

TABLE 2.—*Summary of cytotoxic tests*

		Number of patients showing								
		>20%					>30%			
		Cell reduction								
		Series I and II					Series III			
		Lymphocyte: target cell					Lymphocyte: target cell			
Patients	Target cells	Number of patients	200:1	100:1	50:1	25:1	Number of patients	200:1	100:1	50:1	25:1
Control	587	10	4 *40**	2 *20*	2 *20*	1 *10*					
SAH	glia	1	1	1	1	1					
Glioma		15	6 *40*	6 *40*	3 *20*	1 *7*					
Control	336	7	2 *29*	1 *14*	0	0					
Glioma	glia	14	5 *36*	4 *29*	2 *14*	2 *14*					
Control	622						7	1 *14*	0	0	0
SAH	glia						6	2 *33*	0	0	0
Glioma							16	3 *19*	3 *19*	1 *6*	0
Control	2T	9	3 *30*	1 *11*	1 *11*	1 *11*	6	1 *17*	0	1 *17*	1 *17*
SAH	osteosarcoma	1	1	1	1	1	6	3 *50*	3 *50*	1 *17*	1 *17*
Glioma		15	12 *80*	5 *33*	4 *27*	2 *13*	13	9 *69*	7 *54*	2 *15*	0
Control	118	13	5 *38*	5 *38*	2 *15*	0	4	1 *25*	0	0	0
SAH	glioma	2	2	2	1	0	3	1	1	0	0
Glioma		17	16 *94*	16 *94*	14 *82*	8 *47*	12	8 *67*	4 *33*	3 *25*	0
Control	138	17	7 *41*	5 *29*	0	0	7	2 *28*	1 *14*	1 *14*	0
SAH	glioma	3	3	2	2	0	6	4 *66*	3 *50*	1 *17*	0
Glioma		17	17 *100*	17 *100*	12 *70*	10 *59*	15	2 *13*	1 *6*	0	0
Control	178						3	0	0	0	0
SAH	glioma						2	0	0	0	0
Glioma							9	1 *11*	0	0	0

*Percentage.

cyte target cell ratio the number of cases in series I and II which had >20% target cell reduction. A difference between the tumor and the control patients was found not only for the tumor target cells but also for the osteosarcoma target cells. A glioma-specific component could have been present, however, if one considers the effects obtained with less lymphocytes. The lack of clear tumor specificity was indicated also by the reactivities of the patients with SAH. In series III, therefore, additional SAH patients were included. The new series was conducted with a new glia line (622) and included another glioma line (178). While the protocol was not substantially changed, one of us (H.S.) has not participated in the performance of the tests of series III and a new batch of target cell lines had been obtained from Dr. Pontén. Since titration curves sometimes fluctuated up to 30% cell reduction, this value was taken for the limit of positive effects. The results with the osteosarcoma line were similar to those obtained in series I and II and, compared to the tumor lines, the new glia line was similar to the previous glia lines which were less often affected. In view of the difference in the chosen limit of sensitivity between the two series, the results for glioma line 118 were also similar; however, glioma line 138 was less often affected in this series. The new glioma line 178 was the least sensitive of all lines. The cytotoxic effect of lymphocytes from SAH patients obtained in series I was substantiated on 6 additional patients.

While the effects exerted by individual patients varied considerably, information about the sensitivity of the cell lines could be obtained by the calculation of the mean of the cytotoxicities for each patient group at each ratio (table 3). Glioma line 178 was usually stimulated by the lymphocytes. Since the reactivity with osteosarcoma line 2T was similar in both series, the difference seen with regard to the glioma line 138 might have indicated alteration of the line rather than some accidental change in technical details. As already mentioned, the glioma lines were discontinued and new samples were obtained from Dr. Pontén at the start of series III.

The reactivities of some patients are evaluated in table 4. Tests which gave clear titration effects were regarded positive even if the reduction in cell number compared to that of the medium control was low. If only the results obtained from the glioma line 178 were considered—with the possible justification of the difference between series I and III and the assumption that glioma line 178 does not possess the antigen—they would reveal a glioma-specific pattern. The reactive controls usually affected all cell lines while glioma patients showed more selectivity. The cytotoxicities against the osteosarcoma line disturbed the glioma-specific interpretation, however.

In a number of cases, pregnancy or previous transfusion could explain the effects obtained; but often such factors were not known to be present (table 5). On the other hand, if they were present in the case history, they did not necessarily lead to cytotoxic effects even when 5 cell lines from different donors were used.

DISCUSSION

Tumor-specific reactivity of lymphocytes from glioma patients was reported by Ciembroniewitz and Kolar (*1*) and Levy et al. (*2*). Contact with cultured glioma cells led to blast transformation of autologous lymphocytes and degeneration of target cells. These effects were absent with fibroblast-lymphocyte mixed culture. In the investigation of Levy et al., autologous glioma-specific cytotoxic effects were detected with cross-reactivity between different patients and also with melanoma.

Trouillas (*12*) inoculated glioma patients with an extract of their own tumors. The patients responded with delayed-type hypersensitivity and with production of precipitating antibodies reacting with tumor and also with fetal brain extracts.

Our investigation using allogeneic tests with established lines as targets does not support a tumor- or organ-specific lymphocyte reactivity because of the frequent reactions with the non-brain-derived osteosarcoma line. A number of tests also gave positive results with control patients, and the number was high among the SAH patients. Possibly the poor general condition of these patients influenced the behavior of their lymphocytes. The blood from the glioma patients was often collected on the day of surgery.

TABLE 3.—*Mean percentage of target cell reduction at different lymphocyte-target cell ratios effected by the patient groups*

Patients	Target cells	Series I					Series III				
		Number of patients	Lymphocyte: target cell				Number of patients	Lymphocyte: target cell			
			200:1	100:1	50:1	25:1		200:1	100:1	50:1	25:1
Control	587	8	24±12	15±9	3±7	2±4					
Glioma	glia	12	25±11	18±8	4±6	0±3					
Control	336	4	15	8	3	5					
Glioma	glia	9	20±10	14±7	6±5	−4±3					
Control	622						7	16±6	8±4	−5±9	6±3
SAH	glia						6	28±9	15±7	1±6	3±3
Glioma							16	16±8	19±5	7±4	3±3
Control	2T	6	19±8	3±4	−4±4	−6±4	6	10±10	−2±11	1±10	−14±16
SAH	osteosarcoma	10	41±6	11±5	0±4	−11±5	6	48±13	42±12	22±10	20±7
Glioma							15	38±9	21±7	7±5	−2±4
Control	118	10	43±14	26±9	4±7	2±4	4	5	11	11	−19
SAH	glioma	12	64±11	62±8	46±9	28±8	3	29	19	6	5
Glioma							12	44±29	25±25	4±3	13±9
Control	138	14	35±8	18±6	5±3	2±5	7	7±15	8±6	3±10	−3±8
SAH	glioma	12	64±6	57±6	42±6	30±6	6	51±16	33±11	19±8	13±7
Glioma							16	13±7	0±6	−3±6	−7±5
Control	178						3	−29	−30	−24	−39
SAH	glioma						2	2	−21	−12	−3
Glioma							9	−6±9	−13±5	−2±7	−10±9

TABLE 4.—*Evaluation of individual patients*

Patients								Evaluation of results*					Percentage cell reduction at 200:1		
Date Series I	Number	Sex	Age (yr)	Diagnosis†	Surgery‡	Transfusion	Pregnancy	587	336	2T	118	138	336	2T	118
									Glia	Osteosarcoma	Glioma				
10/3/71	14	♂	46	Astrocytoma III–IV§	—	–		–		+	+	+		60	29→
10/9/71	16	♀	57	Astrocytoma IV	—	–	1	+		+	–	+		16→	23
1/11/72	42	♂	57	Astrocytoma III	—	–		–	–	+	+	–	–3	30	36
1/11/72	45	♀	63	Astrocytoma III	—	–		+	–	+	+	+	–10	30	91
12/21/71	35	♂	31	Astrocytoma I	—	–		+	+		+	+	40		92
1/18/72	48	♂	40	Astrocytoma II	5 weeks	+		–		+	+	+		54	91
1/25/72	52	♀	49	Astrocytoma IV	—	10 years	0	–	–	–	+	+	–6	20	96
12/21/71	39	♂	60	Astrocytoma III–IV	3 weeks	+		+	+	+	+	+	67	65	100
1/11/71	22	♀	68	Astrocytoma IV	3 weeks	+	1	+	+	+	+	+	52	29→	95
12/14/71	44	♀	17	Astrocytoma III	4 weeks	+	0	–	–	–	+	+	11	20	50
1/25/72	36	♀	58	Astrocytoma ?	2 weeks	–	2		+		+	+	39		97
1/25/72	50	♂	66	Astrocytoma II	4 weeks	+		–	–	+	–	+	7	58	13
10/3/71	15	♂	51	SAH		–					+	+			92
10/15/71	18	♂	49	SAH		–					+	+			38
11/26/72	30	♂	60	Intracerebral hematoma		–		+		+		+		81	
9/10/71	11	♀	55	Trigeminal neuralgia		–	1				–	–			–3
9/17/71	12	♂	51	Herniated disk		–					–	–			0
9/24/71	13	♂	20	Spinal cord injury		–					–	–			20
10/22/71	20	♂	53	Herniated disk		–					+	–			35
10/29/71	21	♀	40	Obsessive neurosis		–					+	–			70
12/3/71	34	♂	39	Obsessive neurosis		–		–		–		+		0	
12/14/71	37	♀	32	Trigeminal neuralgia		–	0	+	+		+	+	28→		91
1/11/72	43	♂	57	Brain injury		–		+	–	–	+	+	8	10	31→
1/18/72	46	♂	67	Hydrocephalus		–		–		+	+	+		21→	76
10/9/71	17	♂	23	Obsessive neurosis		–					–	+			17
Series II								587	622	2T	118	138	622	2T	118
2/15/72	56	♂	57	Astrocytoma I	—	–		–		+	+	+		53	68
2/15/72	57	♀	63	Astrocytoma II	—	–	0	–		–	–	+		–8	–4
2/22/72	58	♀	70	Astrocytoma IV	—	–		+		–	+	+		–1	77
3/7/72	60	♂	63	Astrocytoma III	—	–			+	+	+	+	89	92	94
3/7/72	61	♀	43	Astrocytoma III–IV	—	–	1		+	+	+	+	71	97	87
2/15/72	55	♂	41	Obsessive neurosis	—			–		–	–	–		5	–23
2/22/72	59	♀	44	Obsessive neurosis	—		2	–		–	–	–		14	–24
3/7/72	62	♀	51	Obsessive neurosis		4 years	4		+	+	+	+	72	98	80
2/1/72	54	♂	32	Brain injury	—			+			+	+			49

Series III								622	2T	118	138	178	622	2T	118
3/28/72	68	♂	54	Astrocytoma I–II	—	−		−	−		−		−11	16	
4/5/72	72	♂	43	Astrocytoma III	—	−		−	+	+	−		14	45	8→
4/27/72	85	♂	21	Pinealoma	—	−		−	+	+	−	−	−4	52	55
4/5/72	73	♂	50	III	—	−		+	+	+	+		81	93	78
4/27/72	77	♂	56	II	—	−		−			−		28		
4/5/72	86	♂	78	III	—	−		−	−	+	−	−	20	15	74
5/3/72	83	♂	51	II	—	−		−		+	−	−	17		68
3/5/72	81	♀	58	IV	—	−	1	−	−	+	−	−	−5	17	39
12/4/72	76	♀	41	IV	+	−	1	−	+		−		10	40	
5/3/72	36	♀	58	?	16 weeks	−	2	−	−	−	−	−	−17	36	14
4/12/72	71	♂	56	III	4 weeks	+		+	+	+	−		58	49	71
5/9/72	88	♂	43	II	3 years	+		−	+	+	−	−	8	48	59
5/9/72	35	♂	31	I	24 weeks	+		−	+	+	−	−	29	74	45
5/9/72	90	♂	63	II	4 weeks	+		−	+	−	−	−	−40	45	0
3/5/72	45	♀	62	III§	16 weeks	+	1	−	+	+	−	−	16	32	52
3/28/72	67	♀	58	SAH	—	−	0	+	+		+		29→	56	
4/5/72	69	♂	17	Brain injury§	—	−		+	+	+	+		51	61	53
3/28/72	66	♂	45	SAH§	—	−		+	+		+		53	98	
4/12/72	74	♀	71	SAH§	—	−	1	−	−		+		16	27	
4/27/72	82	♀	72	SAH§	—	−	2	−	−	−	−	−	5	30	14
5/3/72	87	♀	32	AVM	—	−	3	−	−	−	−	−	12	10	3
4/5/72	70	♀	51	Trigeminal neuralgia	—	+		−	−	−	+		16	15	−25
4/12/72	65	♂	29	Obsessive neurosis	—	−		−	−		+		26	22	
3/28/72	75	♀	31	Obsessive neurosis	—	−	2	+	−		−		34	22	
4/20/72	78	♀	63	Trigeminal neuralgia	—	−	2	−			−		19		
4/27/72	84	♂	61	Trigeminal neuralgia	—	−		−	−	−	−	−	−12	8	2
5/9/72	89	♀	40	Obsessive neurosis	—	−	2	−	+	−	−	−	16	33	5
4/25/72	80	♂	34	Obsessive neurosis	—	−		−	−	−	−	−	11	−34	7

*Tests with clear titration effect were considered positive (stimulatory effect of lymphocytes at low ratios indicated by →).
†AMV arteriovenous malformation.
‡Time relation to the test.
§Repeat test with closely similar results after 2 weeks.

TABLE 5.—*Summary of all cytotoxic tests*

Patients	Target cells						
	622	336	587	2 T	118	138	178
		Glia		Osteosarcoma		Glioma	
Glioma	5/18* *28*† (2)‡	5/14 *36* (1)	6/15 *40* (2)	21/28 *75* (9)	24/29 *83* (9)	17/32 *53* (7)	0/9
SAH	3/6 (3)		1/1 (1)	4/7 *57* (4)	4/7 *57* (3)	7/9 *77* (6)	0/2
Controls	2/8 *24* (1)	2/7 *29* (1)	4/10 *40* (1)	4/15 *26* (2)	7/15 *47* (3)	11/24 *45* (6)	0/3

*Number of positive cases/No. of tested cases.
Positive cases: >30% target cell reduction or clear titration effect.
† Percentage positive.
‡ No known pregnancy, no transfusion.

We have now learned that lymphocytes from patients on the day of surgery usually incorporate thymidine with a higher rate when compared to controls (*13*). The effect varies, but, in view of the cytotoxicity of activated lymphocytes, this factor may also be important (*14*).

Thus important questions are whether the reactivities reflect antigenic differences and, if so, what the nature of antigen is. While it is possible in some patients that the activity of the lymphocytes is due to presensitization to isoantigens, in others the lack of pregnancy and surgery, i.e., blood transfusion, would exclude presensitization. Sensitization in vitro may occur; however, it has not been demonstrated as early as 48 hours (*5*). Therefore, if the effects we obtained were due to in vitro sensitization to isoantigens, we would have to assume that the conditions of this test allow earlier detection of this phenomenon than has been reported previously.

Autosensitization could possibly cause the cytotoxicity of these lymphocytes, conceivably initiated by traumatic or inflammatory events. This possibility seems plausible in view of the results obtained with the lymphocytes from patients with SAH.

With regard to the problems of evaluating the results, we feel that the use of several concentrations of lymphocytes is essential, since it helps to judge the results in which lymphocytes are seen to stimulate and to reveal cases in which non-lymphocyte-dependent factors may lower cell numbers.

With our panel of cell lines, normal cell-derived lines may be less sensitive to cytotoxicity—a factor which, if left unnoticed, may lead to faulty tumor-specific interpretations.

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PART I

CYTOTOXICITY ASSAYS

DISCUSSION

OUTLINE OF DISCUSSION—CYTOTOXICITY ASSAYS

I. Target cells

A. Fresh cells
1. Preparation—mechanical, enzymes
2. Preservation—short-term: room temperature versus 4° C; long-term: techniques for liquid nitrogen storage
3. Characterization
4. Controls: What to use and how much of appropriate type of cell is represented? Other tumor cells versus normal cells from same tissue

B. Tissue culture
1. Primary, early passage, or established—advantages and disadvantages of each: evidence for loss or gain of antigens in culture
2. Culture conditions—techniques used to grow cells, use of viral transformation
3. Characterization—histology, cytology, electron microscopy, biochemical, antigenic, HL-A, karyotypes
4. What types are available?—exchange of cultures among laboratories
5. Mycoplasma and other contaminants—use of antibiotics; control procedures
6. Control cultures
   a. Suitability of skin fibroblasts
   b. What other control cultures should be used—normal cells from same tissue, other tissue-culture tumor lines

II. Attacker lymphocytes

A. Peripheral blood
1. Methods for separation of lymphocytes
2. Degree of purity of preparation
3. Treatment of attackers with distilled water, Boyle's solution; preservation of attackers at different temperatures, including freezing
4. Effects of platelets, granulocytes, and other contaminants
5. Results of comparisons between different types of preparation

B. Other sources: regional lymph nodes, other nodes, spleen

III. Assay method

A. Media
1. Differences between various media
2. Types and concentration of serum used: human versus fetal bovine serum

B. Size of wells or tubes

C. Visual versus radiolabels: What is being measured?

D. Other variables in assay:
1. Length of incubation
2. pH, presence of CO_2
3. Rocking
4. Attackers added to cells in suspension or on monolayers
5. Removal of nonspecific cells during incubation

E. Attacker/target cell ratios used
1. Results of dose-response curves
2. Basis of calculation: added cells versus plated target cells

F. Methods for calculating results
1. Background: media control, normals, noneffector cell, autologous
2. Formula used: true percent cytotoxicity, or other

G. Design of individual experiments
1. Size of checkerboard used: number of control attackers and target cells
2. Positive and negative controls: What is adequate?

H. Specificity—methods for defining
   1. Tests against other cells
   2. Inhibition assays—whole cells, antigens
I. Reproducibility of assays
   1. Results of repeat of same test on different days
   2. "Technical failures"—What are they and how often do they occur?
   3. Logistics and problems with serial measurements on patients
J. Nature of attacker lymphocytes
   1. B- or T-cells
   2. Effects of drugs, X-ray, etc.

IV. Serum blocking
   A. Design of experiments—serum left in during incubation or preincubation with target or attacker cells
   B. Effects of normal serum:—versus no human serum—Are there some normal sera which inhibit more than patients?
   C. Results at varying concentrations of serum
   D. Characterization of blocking factors
   E. Relationship of blocking effects to clinical state of patients

V. Unblocking factors: same series of questions as for blocking

R. Herberman: As several speakers have already stressed, the nature of the target cells being used for the various types of cytotoxicity tests can make an important difference in the results. The discussion has been arbitrarily divided into consideration of fresh cells, taken directly from the patient, as targets, followed by tissue-culture target cells.

First with fresh cells, what factors have been important in the preparation of the cells? What problems have been noted with mechanical versus chemical dissociation of cells? What are the best ways to get cells with high viability?

J. McCoy: We tried a variety of fresh solid-tumor materials, including lymphosarcoma, Hodgkin's disease, and on occasions other solid tumors, and ran into a number of problems. Frequently the initial viability of the dissociated tumor cells was too low to be suitable for our particular tests, the ^{51}chromium (^{51}Cr)-release lymphocyte cytotoxicity assay. The second major problem was how to get single cell suspensions for use as targets, because of clumping of cells. However, table 1 gives results of a good positive test that we ran, using fresh lymphosarcoma cells. The patient's autologous peripheral lymphocytes were run against labeled tumor cells that pathologically contained lymphosarcoma cells, and also against spleen cells, which had no evidence of tumor. Reactivity was good when autologous lymphoyctes were tested against the labeled lymphosarcoma cells, but no activity was seen against the normal spleen cell targets. However, we had trouble getting good viable cells that would not aggregate during our 4-hour incubation period. We did not attempt any density-gradient separation of viable from nonviable cells, but this might be helpful for preparing cells for our assay.

Herberman: All your results have been obtained with mechanical dissociation of the cells rather than with enzymatic treatment?

McCoy: Yes. At this point we have not methodically investigated methods of enzymatic suspension of cells. Basically this is just a mechanical disruption of the tissues.

Herberman: Does anybody else want to comment on cytotoxicity tests they have done with fresh cells?

G. Santos: I wonder why nobody answers this question.

TABLE 1.—*Lymphocyte cytotoxicity reactions of patient and unrelated individuals against cells of patient, K.E., with lymphoma*

Attacking lymphocytes	Cytotoxicity (±SE)		
	Labeled tumor cells	Labeled normal spleen cells	Labeled allogeneic normal lymphocyte
Patient, K.E.			
Peripheral lymphocytes	22.0 (0.9)*	−0.6 (0.5)	−0.6 (0.6)
Spleen cells	3.8 (0.7)*	0	−0.7 (0.5)
Allogeneic lymphoma patient	7.5 (0.7)*	0.9 (1.1)	−0.5 (0.4)
Normal control #1	3.3 (0.9)*	0.1 (0.6)	0
#2	8.3 (1.0)*	−1.7 (0.6)	0.8 (0.6)
#3	1.2 (0.7)	−0.2 (0.5)	0.4 (0.5)

*Percent lysis significantly different from background release, $P<0.05$.

We have done a few tests and had the same problem. I presume either one has heard it is a problem and so avoids it or one has tried it without success.

Herberman: We should raise a question about the general assumption that, for cytotoxicity studies with solid tumors, fresh cells won't work and that tissue culture cells are needed. How many people who have been working on these assays have actually attempted to use cells directly from the patients for microcytotoxicity or other assays? For instance, could fresh target cells be used in assays that are not dependent on a high proliferative capacity? Perhaps Dr. Bean's proline assay would work with fresh cells.

M. Byrne: We don't use fresh tumor cells; we use cells that have been grown in monolayer. Most of our experiments are done with first- to sixth-passage cells, except for those with the melanoma cell line BERO.

P. Koldovsky: We don't use freshly obtained cells either. There is another problem connected with using material directly from the patient. Dr. I. Witz demonstrated that tumors can be coated in vivo by blocking antibodies. Presence of this type of antibodies can completely abolish finding of cell-mediated cytotoxicity.

Herberman: It's probably fair to say from the lack of response on this question that it may be more a theoretical objection rather than one supported by experimental data.

J. Gutterman: I don't see the objection to doing the cytotoxicity test if the fresh cells are already coated with immunoglobulin. You don't know if antibody on the cell surface prevents cytotoxicity. This is a question to be answered.

Herberman: The other side of the point is the demonstration of lymphocyte-dependent antibody. One might expect that, if target cells are coated with such antibody, test lymphocytes would cause a fair amount of cytotoxicity.

C. McKhann: By the existing ground rules, to prove or demonstrate blocking capacity or enhancing antibody, you have to have a cell that is killed by lymphocytes in the first place. If you don't have that, you don't have a baseline for the rest of your assay. Another thing is the converse of this. One problem that should be considered is when the culture is harvested for use. As demonstrated by Dr. Cikes in Klein's group, the antigenicity of the cells may go down quite a bit, particularly in a very proliferative phase of culture. Thus when you subculture you may have cells that are relatively, at least in the whole population of cells, less antigenic than they would be when they're pretty well crowded out.

G. Mavligit: To go back to the viability problem, using fresh tumor biopsies, we can separate viable from nonviable tumor cells. We tried to use viable tumor cells in lymphocyte cytotoxicity assay. Cell kill was measured by trypan-blue dye exclusion. With 97% viable cell population, it was difficult to maintain viable cells in culture even for 1 day. The index of trypan-blue-positive cells went up even without adding lymphocytes, so that the results could not really be evaluated. If we improve the culture conditions, we may still be able to keep those separated viable cells in the culture and use the simple trypan-blue test.

Herberman: What culture media were you using?

Mavligit: Ham, F10.

Santos: Does anyone know of data in which a KCl extract was taken and then put back on an indicator cell, for example, in order to measure cellular immunity. I think there are models for getting around growing the cell.

N. Levy: In regard to blocking of the cells, we did tests on one patient 2 months ago where we eluted, at low pH, primary cells that had not been dispersed by trypsin. We got some blocking activity in the supernatant but never proved that it was an immunoglobulin. These results do illustrate a theoretical disadvantage in use of primary cells, but this disadvantage can easily be circumvented by the cells being treated with trypsin and washed free of adsorbed blocking factors.

Herberman: But trypsin may inactivate or alter the antigens.

Levy: But they should regenerate. Trypsinize the primary cells, seed them in plates, and perform the test the next morning. This is the way most of us using cultured cells are doing the test anyway.

S. Golub: We have not tried to compare enzymatic and mechanical dispersion in our melanoma experiments, but in terms of establishing melanoma biopsies in culture our impression has been that enzymatic dispersion tends to yield cultures with a higher contamination of fibroblasts, so we primarily use mechanical means. If this is true, then there are certain dangers inherent in using the enzymatic dispersion for primary cultures.

Herberman: This certainly seems to be true, for instance, for breast cancer, as Lasfargues has shown using the spilling technique of slicing finely with a sharp knife. This tends to get the tumor cells out relative to the stromal elements.

M. Sigel: You cannot do the whole procedure because of the low viability and low counts of the original cells. Would you at least try a first-step technique? Assume that in the killing effect there is first attachment of lymphocytes to the target cells. Could you use a section of tumor or tumor cell to measure lymphocyte attachment even when killing is difficult to ascertain?

Herberman: Dr. Sinkovics, have you tried this?

J. Sinkovics: We did not do exactly this, but we tried something similar in the late 1960's. We failed and could not come up with such a test earlier. Tumor biopsies or primary cultures of tumor were trypsinized. The cells were seeded on the coverslips of Leighton tubes and were exposed to the patient's own lymphocytes. The result was that normal lymphocytes damaged these primary cultures of tumors better than the patient's own lymphocytes. We concluded that probably the primary cultures of tumors did not express tumor antigens very well because they were derived from an immunologic hostile environment; i.e., they were under immunologic attack in the

patient. Later at our Institute, Romsdahl and Cox[1] eluted blocking factors from primary sarcomas and melanomas. A few days after we seeded cells in Leighton tubes, we thought that most of them looked like fibroblasts and not tumor cells; although small colonies of tumor cells appeared, most of the cells appeared fibroblastic. Our first success in showing a specific cytotoxic effect was when we established a chondrosarcoma in tissue culture and the patient was still alive. The lymphocytes of this patient specifically destroyed his tumor cells without damaging his own fibroblasts.[2] That was in 1969. Since then, instead of primary cultures of tumors we have used established tumor cell lines as target cells.[3]

W. J. Martin: We have not been able to demonstrate the presence in tumor-bearing animals of cytotoxic lymphoid cells against freshly explanted autologous tumor cells. Viable tumor-cell suspensions can be obtained by Pronase digestion of teased solid tumors. Pronase treatment does not appear to affect the ability of tumor cells to be lysed by cytotoxic lymphocytes directed against H-2 alloantigens. Pronase treatment may, however, interfere with the expression of tumor-specific antigens.

Herberman: Since most of the work with fresh cells has been with the ^{51}Cr-release assays, one question is: Can one use the tumor cells over a period of time or does the test have to be done on the day of surgery? This brings up the problem of preservation. What have been the problems or success with preserving the tumor cells?

McCoy: We have found that the quicker we can get and prepare the material, the better off the viability will be. But even with rapidly processed material, the viability of tumors such as Hodgkin's and lymphomas, is frequently below 50–70%. These obviously are inadequate for our tests. Trials at slow-freezing these materials as single cell suspensions and later thawing to obtain viable cells have been unsuccessful.

Herberman: Are you saying that you have not been able to viable-cell-freeze any of the target cells that you are using?

McCoy: No, we have been able to viable-cell-freeze fresh leukemia cells with no problem even when they are 24 hours old. The viability after freezing and storing leukemia cells for long periods is still quite high. Frozen cells are quite adequate for lymphoycte cytotoxicity tests and also seem to maintain their antigenicity after long-term storage.

Koldovsky: In animal solid tumors, it was established that usually not more than 5–10% of the cells are alive—with the use of the dye-exclusion technique. Has anybody studied freshly obtained human tumors in this respect?

Mavligit: In our experiments, viable cell population in human solid tumors after mechanical dispersion into a single cell suspension varied from 2 to 97%, depending on the tumor. Lymphoid tumors tend to be more viable than other tumors like colon carcinoma or lung cancer. Especially in lung cancer, there is frequently a lot of necrosis, and one can expect to see a lot of nonviable cells as judged from the trypan-blue test. I say nonviable cells without knowing whether they are really dead or just damaged.

Herberman: That is a rather difficult question to answer. To do the cell counts, you have to dissociate the cells.

Mavligit: That is correct. To get a single cell suspension, I have been sieving those cells through a 60-mesh screen and found that this adds to the damage. At present, I cut and chop the tumor delicately with a scapel and let the chunks settle, which results in a more viable single cell suspension.

Herberman: But what is the yield?

Mavligit: Depending on the cellularity of the tumor, millions of cells can be obtained from a tumor that is about $2\times2\times2$ cm, sometimes >200 million cells. Sarcoma, for instance, would give less yield than adenocarcinoma.

Levy: It is hazardous to use trypan-blue exclusion as an index of viability with nonlymphoid cells. I have seen suspensions of epithelioid cells, 100% nonviable by trypan-blue exclusion, that had plating efficiencies of 20–60%. Dr. Mavligit's finding of greater viability among lymphoid than epithelioid cells is consistent with my observations. In response to Dr. Koldovsky's question, we have never actually looked at the viability of fresh tumor cells but do generally get plating efficiencies of 3–10% with primary tumor cells dispersed either by trypsin or mechanical means.

McKhann: Even with animal tumors, the amount of killing you get by pressing through a sieve is enormous compared to the amount you get by trypsinizing the tumor. This occurs even when obvious necrotic tumor is excluded.

Herberman: Let's go on to the next point, about characterization. I would like to stress that it is important with any of the target cells, particularly from various solid tumors, to know as precisely as possible the percentage of tumor cells with which one is dealing. Dr. McCoy's data on a lymphosarcoma patient demonstrate that the part of the spleen with pathologic evidence of tumor showed positive results. Yet, another part of the spleen, morphologically normal, gave negative results. Since there's no further comment on this, let's raise the last problem regarding the fresh target cells: What cells should be used as controls for the tumor cells? For instance, if one is doing a cytotoxicity test with leukemic or lymphoma cells, what is the appropriate cell type to use as the control and has this generally been used?

Santos: Although I don't know the answer, with a

[1] Romsdahl M, Cox I: Evidence for enhancing antibodies in human sarcomas. Proc Am Assoc Cancer Res 12:66, 1971.

[2] Sinkovics J, Shirato E, Martin R, et al: Chondrosarcoma. Immune reactions of a patient to autologous tumor. Cancer 27:782–793, 1971.

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lymphatic leukemia patient, I suppose you could wait until the patient is in remission and then run the test against his own normal lymphocytes, or his phytohemagglutinin (PHA)-transformed lymphocytes.

A. Hollinshead: Remission cells are a good idea, but I'm wondering whether we shouldn't pay more attention to bone marrow cells, since blast cells might have antigenic similarities to immature forms. Also, in regard to counting the cells, we routinely use neutral red, trypan blue, and white blood cell diluent and have indexes for our solid tumors and their control tissues. In general, with our techniques, the counterpart normal tissues yield fewer cells/cm² crude material than the tumors.

McCoy: If one is dealing with a population of cells high in blasts, what is the proper control? There's always the possibility that these cells are just more fragile than normal lymphocytes and, therefore, when one is running blasts and normal lymphocytes as controls, the blasts may be more susceptible to spontaneous death during the assay. Our data from the identical twin study argue against this idea. When we used an autologous control (i.e., unlabeled autologous blasts with blasts that are labeled), usually we found quite low spontaneous ^{51}Cr-release backgrounds. We also had bone marrow from normal and remission patients in our identical twin study. There were a series of tests in which the bone marrow blasts were quite susceptible to lysis, whereas the bone marrow from the normal identical twin was not. If bone marrow represents good control material, then our concept of immunologic specificity is strengthened.

Santos: Except that the normal marrow doesn't have all that many blasts. If somebody can remove those few percent of blasts and concentrate them very nicely without damaging them, I'd like to hear about it.

Herberman: What about the control of using PHA-stimulated lymphocytes? Is this an adequate control? Are these blasts acceptable as a good control for lymphoblasts?

Gutterman: Leukemic blasts are not stimulated by PHA. When we do leukapheresis from a patient who comes into the hospital with 90% blasts, there are a certain number of normal lymphocytes, so we may get minimal stimulation. But, in general, the leukemic cell populations are not turned on by PHA or other mitogens.

Golub: You also have a problem in that type of experiment; PHA-stimulated cells may produce lymphotoxins that can affect your effector cells.

Mavligit: PHA-stimulated and washed lymphocytes can stimulate autologous lymphocytes in mixed leukocyte culture (MLC).[4] Stimulation with PHA probably alters their antigenicity. I believe that this is a good control.

Herberman: Let's proceed to tissue-culture target cells. One of my first questions, which Dr. Levy and some others have already raised, was: When should one use the tissue culture cells? One can use a very early passage or an established line. What is the actual evidence that antigens are gained upon long-term passage or are altered or lost during the culture?

E. Klein: Is it possible to make a general statement? Cell lines may express new antigens or may lose antigens. One has to be particularly aware that changes may occur in culture lines. Besides permanent changes, there is evidence that expression of surface antigens depends on culture conditions. Starved cells have a lower concentration. In monolayer cultures, judged by the appearance of mixed hemadsorption, the antigen concentration is high at the edges and processes; confluent cultures give less effective reactions.

Herberman: Dr. O'Toole, you've been using both early passage and established lines of bladder carcinoma. What is your feeling on this?

C. O'Toole: I have used primary cultures from the second passage onward to eliminate leukocytes often present in fresh tumor material; these leukocytes may stimulate the effector cells in an allogeneic cytotoxicity test system. No major differences have emerged between the cytotoxicity seen on primary cultures and established lines of the same tumor type. Established lines seem more sensitive, however, than primary material to specific cytotoxicity.

M. Takasugi: We have used both short-term and established lines. We get clearer specificity with short-term cultures but also get some specificity with long-term lines. A problem you face with established cultures is the higher reactivity with normal lymphocytes. Scores of the percentage killed are much higher with established lines than with short-term cultures.

McKhann: Has anybody worked with a good bona fide culture line and seen it lose its responsiveness to lymphocytes over a period of time?

Levy: Yes. I presented data earlier showing that 2 of 3 lines tested did, with repeated passage, lose their sensitivity to immune lymphocytes. We have also seen many lines lose their sensitivity to anti-HL-A isoantisera after several passages.

Herberman: Were they cloned lines?

Levy: Everything that we used was cloned. They were tested first in the 30th passage. One line must be over the 100th passage by now, and it is still an effective target cell for cell-mediated reactions. We don't know whether it's as good as the initial primary cells.

D. Rubin: The question of antigen loss on target cells after in vitro cultivation is more pertinent to the nature of immunotherapy which may be considered on the basis of these laboratory studies. Dr. Sinkovics believes there is an advantage in studying these cells after growing them in the absence of immunoselection (in vitro) because other antigens or covert antigens may be expressed. It seems to me that the fresh tumor cells from the patient —cells that have escaped immune destruction—should be extensively characterized with regard to their antigenic composition. In other words, if the culture line retains a certain antigenicity after prolonged passage, the antigens detected by these methods may not necessarily be the ones

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on which an effective form of immunotherapy can be used.

Levy: I know of no evidence for such antigenic modulation in solid tumors. Such evidence would be a prerequisite to a discussion of in vitro "cell selection."

Sinkovics: We had a patient whose tumors were biopsied several times, set in primary cultures, and tested with his lymphocytes. The majority of the cells were fibroblasts and the minority were what we thought were chondrosarcoma cells. With this target cell system, we could not detect any specific reaction. From a few colonies of chondrosarcoma cells, we established a culture. From then on, every time we tested this man, his lymphocytes destroyed the autologous, established tumor-cell line. He also had antibodies demonstrable with the indirect immunofluorescent technique, as shown by Dr. E. Shirato, against his established cell line. So we are using his serum as a standard serum against sarcoma cultures; and whenever that serum is positive with a sarcoma culture, we conclude that the sarcoma culture carried sarcoma antigens and that would then be a culture selected as target cells in the cytotoxicity test with lymphocytes.[5]

A. Hollinshead: We followed a hamster adenotumor for a long time and tested it both in vivo and in vitro for the adeno-induced cell-membrane-soluble antigen.[6] At about passage 32, not many tumors were produced and the antigen titer diminished. At about passage 41, we didn't get any antigen off the membrane, no tumors were produced, and the ploidy had changed. I wonder whether anyone has followed the changes in aneuploidy, diploidy, and polyploidy patterns.

M. Takasugi: There is another difference between short-term and long-term cultures. We can usually type short-term cultures for HL-A antigens and the results agree with lymphocyte typing, but typing of long-term cultures is somewhat difficult. It's hard to find clear specificities on these cells. Dr. E. Plata found that the cells we had difficulty in HL-A typing are triploid. Perhaps this disturbs some of the HL-A antigens on the surface of the cells so that we cannot test for them.

R. Oldham: There are theoretical problems with any type of cell line you use, but I wonder if we ought not think about the practical aspects. If you use early-passage cells from lung, breast, or colon carcinomas, you will have a shortage of target cells available for studies most of the time. I don't think most people's experiences are those of Dr. Levy when it comes to these common tumors of man. I don't think a 25% success rate in growing tumors from explant is anywhere near what you'll find with these tumors. Consequently, for the purpose of exchanging target cells between laboratories to have large quantities of cells available for study, it is not going to be feasible to look at either primary explant cells or early-passage lines as target cells. We will be forced to use established lines more and more in the future.

Herberman: Has anyone, besides Dr. Levy, had experience with bringing out early passages from the freezer?

McKhann: It should be made clear that there are great differences in bringing out early passage cells in primary tumors, because this is what has happened to so many people who have gotten tumors from the Naval Biomedical Laboratory in Oakland, California.

Koldovsky: Within the last year, we prepared several lines of human tumor. These lines underwent between 10 and 25 passages and still we can detect surface tumor-associated antigens. That the tumor-specific transplantation antigen (TSTA) is a very stable characteristic in animal tumors was already shown, e.g., by Sjögren.[7] Normal transplantation antigen can sometimes be lost, like in the case of sarcoma 1. Antigenic changes (mutation) were observed some time ago, the first review being written in 1935.[8] Carcinoembryonic antigen (CEA) in an organ culture lasting 6 years was described by Burtin.[9]

P. Levine: In regard to the stability of antigens on the surface of sarcoma cells in tissue culture, the experience with lines from Burkitt's tumor patients may be relevant. It is quite clear that there is a rise and fall of surface Epstein-Barr virus (EBV)-associated antigens and, as Dr. Klein and his colleagues have shown, the amount of cell-surface antigen can be controlled by metabolic inhibitors. Since there are sera reported to be able to pick up sarcoma cell-surface antigens, I would like to know if any of these lines carried at the University of California at Los Angeles have been followed serologically for sarcoma-associated cell-surface antigens over periods of time.

Oldham: I don't have any experience myself with short-passage lines, but data from Dr. Lasfargue's laboratory indicate that with breast carcinoma your chances of getting short-term, early-passage tumor cells in quantities for testing are quite small. Probably $<10\%$ of the tumors he cultures grow for >4 passages. When he tries to split these early-passage growths, either with trypsinization or mechanical removal, these growths no longer attach and proliferate well. With the Naval Laboratory in Oakland, I do have some experience. I received 30 lines from that laboratory, all of which were labeled as colon, breast, lung, or melanoma lines, and all 30 grew out in my laboratory as purely fibroblastic cell lines.

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[9] Burtin P, Buffe D, Kleist S von, et al: Demonstration of the carcinoembryonic antigen specific for digestive cancers in human tumors maintained in organotypic cultures. Int J Cancer 5:88–95, 1970.

Mavligit: We have heard about deletion of antigens. Has anyone ever noticed the acquisition of new antigens on the surface of tumor cells in cultures after several passages?

J. Cohen: I have not observed the acquisition of new antigens, but I have noted an apparent increase in antigenicity. I had a polyoma-transformed cell line, originating from C57BL/Ka mice, in tissue culture for about 3 months when the cells ceased producing progressively growing tumors in syngeneic recipients. I then studied this apparent change in antigenicity and obtained the following information: 1) This cell line would confer immunity on syngeneic recipients and allow them to reject another tumorigenic polyoma line which readily grew in nonimmunized animals and was positive for the TSTA. 2) Lymph node cells from C57BL/Ka animals immunized with polyoma virus were cytotoxic to this cell line in the microcytotoxicity assay. 3) Spleen and peritoneal cells from C57BL/Ka mice that had received a single intraperitoneal injection of 2×10^6 cells were cytotoxic to allogeneic polyoma ascites cells (YAA-C1) in the ^{51}Cr cell-mediated cytotoxicity assay. To date, this cell line has maintained the above characteristics and is growing well in tissue culture.

G. Klein: Lymphoblastoid cell lines which have EBV-determined surface antigens may lose them permanently or temporarily due to culture conditions. In relation to the biopsy, the cultured lines express the EBV-determined antigens after some time in vitro. On the other hand, a Moloney lymphoma, cultured by Dr. Matko Cikes, had a lower amount of Moloney-specific antigen. When put back in the mouse, it displayed the original amount of antigen. As for gains in antigenicity, infection with viruses, by laboratory contamination, can impose specific antigens.

Golub: In response to the question about gaining antigens, established lymphoblastoid cell lines may show anomalies in HL-A typing, including additional specificities.[10]

Regarding Dr. Levine's question, the problem of variation of antigenic expression in sarcoma cells presents obvious difficulties in analyzing the antigenic systems involved. In the complement-fixation (CF) test, extractable CF antigen can vary in titer up to 32-fold with different passages of the same cells.[11] Similarily, Dr. Bloom reported that sensitivity to cytotoxic antibodies fluctuated in sarcoma cell lines, and these fluctuations were not directly predictable by passage number.[12]

Herberman: Let's get on to the next question about culture conditions. Dr. Levy suggested that the media most people are using are not good. Does anyone have any comment or rebuttal to this?

Takasugi: Dr. Levy, why do some people get good results with media low on that scale? One person who uses a medium rated poor on your scale gets very good results.

Levy: When we started doing microcytotoxicity studies with human glioma cells, we found Waymouth's medium inadequate. Puck's N16 and Eagle's minimal essential media were better. With this information, and by a combination of others' reported experience and our own empirical observations, we developed our present medium formula.

Herberman: Do your data apply only to glioma tumors?

Levy: No. We tested normal fibroblasts, normal glia and melanoma cells, and the glioma cells. Our modified medium was best with all 4 cell types, and RPMI 1640 always worst. There was some minor variation between the different cell types with the other media.

Cohen: What criteria did you use to study the effect of the various media on cell growth other than morphology?

Levy: We looked at 3 things: the plating efficiency, growth rate, and ability to support single cell cloning.

Cohen: Did you use the Falcon Microtest II plates to study plating efficiency?

Levy: The plating efficiency studies were done in microtest plates, the growth rates in 25-cm^2 flasks, and the cloning in culture dishes.

Cohen: The Microtest II plates are used in the microcytotoxicity assay, and the culture conditions in these wells are crucial for the success of the test. Did you study the effects of your media in these plates?

Levy: Not entire studies—just the plating efficiency part.

Herberman: I think Dr. Cohen raised a good question. Conceivably conditions good for the cells in culture flasks may not be optimal in the test.

E. Palaszynski: We have obtained results similar to those of Dr. Cohen. In growing certain cell lines in microtiter plates, we obtained results just the opposite to those of Dr. Levy. RPMI 1640 was very good and Eagle's was one of the poorest in supporting cell growth.

McCoy: Dr. Levy, do you make your own media or do you buy your media from commercial firms? We have found that media from different sources differ strikingly in their ability to support cell growth.

Levy: Originally, we used Gibco media. When someone suggested that our poor results with Waymouth's medium were due to Gibco and not the medium's formula itself, we made our own and got identical results. Our modified media are now prepared in part from Gibco concentrates.

Cohen: Using both Waymouth's medium and Ham's F12, I studied the plating efficiency and growth of several cell lines in the Microtest II plates. Cells grew nicely in the large Falcon flasks in Waymouth's medium, but in the plates our cell lines grew twofold or threefold better in Ham's F12. I have also been using the microtest plates to test various lots of fetal calf serum (FCS) as to their suitability for use in the microcytotoxicity assay. To my surprise, there were large differences between several different lots from the same supplier with refer-

[10] Dick HM, Steel CM: Anomalies in HL-A typing. Lancet 1:1135–1136, 1971.

[11] Ebes R, Morton D, Reisfield RA: Unpublished observations.

[12] Bloom E: Further definition by cytotoxicity tests of cell surface antigens of human sarcomas in culture. Cancer Res 32:960–967, 1972.

ence to their ability to support cell growth in the Microtest II wells.

Sinkovics: We believe that some commercially available sera contain endotoxins. This can now be tested with the Limulus test using polyphemus hemolymph, which clots on contact with nanogram amounts of endotoxins.[13] In sera collected at slaughterhouses, gram-negative rods may grow and release endotoxin. Then the sera are filtered and the product is sterile, but it will contain endotoxin which could be very toxic to cultured cells. However, this could be detected in every batch by a single test for endotoxin.

Herberman: A variety of fetal bovine sera was recently studied.[14] Marked differences were evident between one commercial batch of fetal bovine serum (FBS) and another. Unfortunately, the report of that survey did not disclose which companies did well.

Levine: Even within the same company, there are great differences. Virology laboratories found it necessary to pretest many lots for their ability to support cell growth and for lack of contamination. They then asked the commercial company to save large volumes of the lot that they want to use and keep buying from that lot until it is used up. This arrangement can often keep a laboratory well supplied for more than 6–12 months.

Levy: Elimination of FCS did much to reduce "nonspecific" killing in our microcytotoxicity assay. The ability of endotoxin to activate the terminal complement components makes Dr. Sinkovic's comment quite meaningful.

Golub: On that point, we go through the procedures that Dr. Levine has mentioned; we test a number of lots of FCS. I also tested the same lots for blastogenesis with normal lymphocytes. The lot that best promoted the growth of tissue culture cells also caused the most blastogenesis of lymphocytes. So we're trying to avoid the use of FCS for our lymphocytes.

Herberman: Dr. Koldovsky previously suggested that transformation of cells with simian virus 40 (SV40) might help to get some cell lines established. Would someone like to comment?

Koldovsky: Normal human cells or normal cells in general usually propagate very slowly and have a limited lifespan. But I've made several attempts with SV40 and I've also compared WI38 cells already transformed with SV40. Adding this new antigen by transformation does not change the property of the cells too much; you can use them perfectly as the control cells. You have a fast-propagating permanent line.

Herberman: Do you know if this works with tumor cells?

Koldovsky: I never tried it for tumor cells.

Herberman: What criteria can be used to characterize tissue cultures derived from tumor cells to be sure that tumor cells are in culture resembling those in the original biopsy?

Levy: Some cells, such as melanoma cells, possess morphologic markers. Glial cells often have a unique antigenic marker called S100, which is easily detected by fluorescence. Electron microscopy may also reveal 90-A filaments in the cytoplasm of glial cells. We have used these markers to distinguish glial cells from fibroblasts but know of no way to distinguish normal and neoplastic cells in their early culture passages. Classic tissue-culture criteria for tumor cells, such as the formation of colonies in soft agar, are useful only with long-term tumor lines. We even tried inoculating cells into hamster cheek pouches and got as many tumors with normal as with neoplastic human glial cells. Karyotyping is also not helpful in the early passages when diploid cells are generally selected in both normal and neoplastic cell cultures. Not until the tumor cell cultures are well established does polyploidy become dominant.

M. Bean: I'd like to ask Dr. Levy about cloned tumor cells. Some melanomas we put into culture contained melanin for the first 2 or 3 passages. We felt quite comfortable about this until the cells lost the melanin in later passages. In your cultures, where you're cloning early, you should have cloned out some single cells that have melanin in them. Isn't that a sufficient marker, even if the culture later loses the ability to produce melanin?

Levy: Yes, with melanoma you can do this because of the obvious marker. With other tumors it becomes more difficult because cell morphology is so poor a marker. Fibroblasts are generally the cell one wants to exclude, and I have some horrifying photomicrographs of skin fibroblasts assuming myriads of different shapes including some very epithelioid-like poses! One generally needs chemical or immunologic markers to be sure of a cell's origin.

Bean: Could we backtrack to the methodology and how successful you were with the cloning of cell lines? Also, could you discuss your primary culture technique, since you had more success than most of us?

Levy: The medium is probably the most critical variable. (The techniques are discussed in detail in my manuscript.)

Herberman: Could you put a percentage on your success in cloning?

Levy: We are successful in 50–70% of our cloning attempts.

Golub: Do you use conditioned media?

Levy: Yes, we do. When we feed our cells, we routinely remove three-fourths of the medium and leave about one-fourth "conditioned" medium.

Oldham: Dr. Takasugi, do you have any positive evidence that the tumor cells actually change HL-A type from what they were initially or from what the lymphocytes of the donor were?

Takasugi: No, we have not followed them continuously but they are at least stable over periods of several months when they are very easy to type. We can support the

[13] Levine J, Poore T, Young N, et al: Gram-negative sepsis: Detection of endotoxemia with the limulus test. Ann Intern Med 76:1–7, 1972.

[14] Boone CW, Mantel N, Caruso T Jr, et al: Quality control studies on fetal bovine serum used in tissue culture. In Vitro 7:174–189, 1972.

typing of cultured tumor cells with lymphocyte typing of the same person, but we have difficulty in typing long-term lines.

Oldham: You see no evidence of change, then?

Takasugi: All long-term cultures come from short-term cultures and it would be nice to be able to follow them. We have not done this yet.

Koldovsky: It is very important to prove that the cells you get growing from human tumor are really malignant. Loss of contact is not a sufficient criterion. We are using newborn mice treated with antilymphocyte serum and intraperitoneally injecting large doses of cells (10×10^6) into each animal. With normal cells, we never found any growing tissue. However, with malignant tumor cells, we found solid tumors within 10 days, and these tumors were really heterotransplanted human tumors.

I was thinking of using a technique—described by McPherson—to distinguish in the soft agar that normal cells will not form colonies as tumors will. Could you tell me why I should not try this technique, Dr. Levy?

Levy: We have been unable to get our early-passage tumor cells to form colonies in soft agar, although this technique does work well with long-established cell lines.

Sinkovics: In addition to the melanoma and the glioma cells, it is easy to recognize a rhabdomyosarcoma cell, which has myofibril elements that can be seen with the electron microscope and can be tested for with the indirect immunofluorescence technique with antiserum directed to human myoglobulins. Also, rhabdomyosarcoma cells contain glycogen digestible with diastase.

Sigel: Several years ago we used a technique originally developed by Harry Green, and also used by Helen Toolan, utilizing the brain as an "immunologically priviledged" site for xenogeneic transplants. HeLa and KB cells and chick embryo fibroblasts (CEF) infected by Rous sarcoma virus (RSV) gave rise to tumors after intracerebral transplantation to rat brains. The rats had to be pretreated with radiation (400 R) and cortisone. Apparently the brain is not as "immunologically priviledged" as originally thought. Normal cells grew less efficiently; i.e., it was necessary to inject 100 times more normal CEF than RSV-infected (transformed) CEF. Normal cells formed small, non-neoplastic nests in contrast with the extensive tumorous growths produced by HeLa, KB, or RSV-infected CEF.[15,16,17,18]

I want to make another point not yet mentioned, though it is on the agenda. One should immunize animals with the original tumor cells or with cells in primary or early-passage tissue culture. The antisera should prove very useful for study and comparison of membrane antigens. They would aid in the ascertainment that such antigens are retained on cells in continuous culture. You need many criteria for assessing the integrity of cellular identity, including their immunologic and transplantation properties.

McCoy: To return to the idea about the possible use of semisolid agar for cloning purposes, Dr. Cannon in our laboratory is working on cell cloning. She was able to very nicely clone one of your cell lines, Dr. Levy, the Shailer line. Maybe Dr. Cannon would comment briefly on this. Also, has anyone tried to use the factor-free media reported by Jainchill and Todaro[19] with which they have successfully grown SV40 animal tumor cells? This medium apparently is selective, in that tumor cells will grow but normal cells will not.

Bean: I don't think it's a fair test to take a cell line that's already been cloned and then put it into the soft agar and claim success.

G. Cannon: That is what we have basically done with HT-39 and the lung carcinoma 2563. They are both established lines. We have not been able to do it with early tumor lines.

Rubin: There is another marker for identifying lymphoid cells: the production of the second component of complement. The assays are so sensitive that you can detect very small quantities being produced. Regardless of how they may look in tissue culture, synthesis of C2 is specific for lymphoid cells. Skin fibroblasts, and many other cell types, do not make this second component.

Santos: Dr. Herberman, about culturing leukemic cells, I think the problems are even worse than we've heard here. Would you care to comment?

Herberman: My impression is that lymphoblastoid cultures have been started from leukemia, a Burkitt's lymphoma patient, various other diseases, and from normal individuals. Besides, whether or not they're EBV-positive, we haven't seen any convincing markers of disease relatedness. Maybe Dr. Klein would like to comment.

E. Klein: An excellent marker is the IgM immunoglobulin on the cell surface. It is found on nearly all cells of a number of biopsies of Burkitt's tumor and is usually maintained in the established lines.

Herberman: But is that a marker for EBV?

E. Klein: No. IgM is often present on chronic lymphocytic leukemia cells. This is a marker for the lymphoid cells, also. However, I have not seen high amounts of surface immunoglobulins on non-Burkitt lines. Dr. K. Nilsson in Uppsala has delineated criteria concerning morphology and growth behavior, conditions for establishment of the lines according to which he can distinguish Burkitt cells. The EBV genome is in all lymphoblastoid lines.

[15] Sigel M, Scotti T, Wryk M, et al: Tubule formation in rat brains by monkey kidney cells previously grown in vitro. Nature (Lond) 182:1034–1035, 1958.

[16] Sigel M, Scotti T, Schulz V, et al: Heterologous transplantation for demonstration of the oncogenic effect of Rous sarcoma virus. Cancer Res 20:1338–1340, 1960.

[17] Scotti T, Wryk M, Dorsey M, et al: Transplantation of human malignant epithelial cells from tissue culture to rat brains. Cancer Res 20:58–61, 1960.

[18] Scotti T, Dorsey M, Lefkowitz S, et al: Growth of normal and Rous sarcoma virus-infected chick embryo cells in rat brains. Cancer Res 23:531–534, 1963.

[19] Jainchill J, Todaro G: Stimulation of cell growth in vitro by serum with and without growth factor. Exp Cell Res 59:137–146, 1970.

Koldovsky: On normal lymphocytes, can't you find immunoglobulins too?

E. Klein: Yes, but the biopsy will have about 99% immunoglobulin-positive cells: either μ or k or only μ chains in the different cases, but uniform for a given tumor. The tumor is nearly a homogeneous population, and the same immunoglobulin is most often maintained in the culture.

Herberman: Another type of antigen that could probably be used as a marker for colon carcinoma lines, as suggested by Dr. Koldovsky, is CEA. But to get a better picture of how good a marker it is, has anyone had any experience in finding CEA on noncolonic carcinoma lines?

Levy: First of all, shouldn't we establish if we're really dealing with CEA? That has a pretty definitive connotation.

Oldham: As you know, we sent off 5 lines to be tested for CEA, and the data showed that all cell lines might have a low level of it. Some people have stated that all sera might have a low level of it. In individual testing in our cell lines, anything above 2 ng/ml was positive for CEA and anything below that was a normal amount being produced. Of the 5 cell lines (1 lymphoid, 1 lung, 2 breast, and 1 colon), only the colon line produced high levels of CEA that were >15 ng/ml. Interestingly, the BT20 breast line produced CEA levels at about 2.9, which was above the level of 2, but was still quite a lot lower than the level in the colon. I don't know if anybody really knows what the average production of CEA is for noncolonic, long-term, established lines.

Herberman: The significance of low-level inhibition in the radioimmunoassay for CEA is not clear. Results of 1 or 2 ng might not be CEA, but rather nonspecific inhibition. This test was done by Hansen's technique. Dr. C. Todd at the City of Hope National Medical Center in Duarte, California, tested the same colon carcinoma line and found fairly high amounts. However, he also had a cervical carcinoma line that reacted with his antiserum.[20]

Hollinshead: Dr. Koldovsky and I were talking earlier about this and wondered about the specificity of the goat antisera, whether the test is against any specific structural antigen, and whether these sera are appropriately absorbed. We should wait to think about this as a marker until things have cleared up.

Herberman: The specificity of various antisera to CEA is not yet completely clear. As illustrated by the comments made today, the nature of target cells makes a major difference in the results obtained. Several of the people here have talked about cell lines that gave specific results (if not totally specific, at least partially specific). I would urge a more open exchange of cell lines between the laboratories performing these assays. Even if a particular laboratory is not studying the disease from which a given line was derived, it could be useful as a control.

McKhann: The convenience of using established cell lines, which are well characterized and can be proved to have a common antigen for the particular tumor you are studying, is extraordinary because you can test far more patients rather than trying to rely on the hit and miss of establishing primary cultures. Obviously this has to be checked out over a period of time against primary cultures; you can't really get around that altogether. The real requirements are: a thorough characterization of the lines which have been around (the criteria which have been mentioned already) and also the realization that there will be changes in these lines. A central intelligence or a central control station should be established to track such information so that, when a particular line becomes nonantigenic or something happens to it, the information would be sent to all the people who are using the line. But several laboratories have many lines and a lot of laboratories have at least one line that they have been using quite consistently and it seems to be working very well. Bladder tumors, breast tumors, and things like that at least would form a nucleus with this type of exchange. I'm not thinking of lines that could be perpetuated ad infinitum, but more of having a pretty good melanoma line or two, which could be replaced with a new good melanoma line if it became bad or was lost for some reason. I think this is valuable.

Sigel: I'd like to ask about exchange agencies, for example, the American Type Culture Collection (ATCC), which was organized for the purpose of maintaining and testing tissue cultures. The objective was to receive, maintain, and monitor certain cell lines and to apply the numerous parameters to assure integrity of the line. It would be advantageous to have cultures placed in such a repository rather than impose on the good will of one investigator to supply the whole world.

Herberman: I don't know what the best mechanism might be, but the ATCC is certainly one possibility.

Levy: In principle this is a fine idea, but it has 2 inherent problems. First, all lines should be screened by a central agency for contamination with extraneous cell types, mycoplasma, and other microorganisms and examined for appropriate markers to verify the origin of the cell line. The agency must be as good as the investigator himself would be and must be very critical of the lines it accepts. The other point, and I don't mean to be facetious, is, if all of us are relying on a central agency, then who will start the cell lines! We all know how few good long-term lines emerge from our many attempts.

Herberman: How many of the currently used lines are contaminated with mycoplasma? What criteria should be used to certify a line as being free of mycoplasma? Can one cure mycoplasma-contaminated lines?

Bean: I'll respond to that for Dr. J. Fogh from the Sloan-Kettering Institute for Cancer Research. His personal feeling about this is that he can successfully cure a mycoplasma infection as long as the cell has not been pretreated inadequately. It's almost the same thing that you see with patients when they have been treated with inadequate antibiotic therapy and they develop a re-

[20] Egan ML, Todd CW: Carcinoembryonic antigen: Synthesis by a continuous line of adenocarcinoma cells. J Natl Cancer Inst 49:887–889, 1972.

sistant organism. We have been working with Dr. Fogh, and using his methods, we eliminated mycoplasma contamination as a major laboratory problem. Now, we don't accept any lines containing mycoplasma into our laboratory. The lines go to Dr. Fogh first. He assays several ways for mycoplasma (colony growth and also orcein stain of an infected FL cell culture). The ultimate way to make sure you don't have any mycoplasma is by electron microscopy, but this is not always convenient as an assay. The lines he received from outside that were contaminated with mycoplasma were effectively cleaned up.

Herberman: The cleaning-up has been done with antibiotics, hasn't it?

Bean: Yes.

Golub: Does anybody have definitive information on the effect of mycoplasma on cell-mediated immunity?

Herberman: You mean on the question of whether mycoplasma produces antigens that will be detected in the cellular assays?

Golub: Yes, antigens that sensitized lymphocytes will react against. I have no doubt that people may have antibodies against mycoplasma and that these may affect serum-mediated tests. I don't know what information there is with cell-mediated immunity.

Sinkovics: We described tumor cell lines free of mycoplasma and cell lines artificially contaminated with mycoplasma and tested these against the buffy-coat leukocytes of patients with tumors.[21] We found that the lymphocytes would not get to the mycoplasma-coated target cells, but macrophages and granulocytes would; nonspecific, slow, protracted cell destruction occurred, which was far different from what we see when we observe lymphocyte-mediated cytotoxicity. There were some human sera which very actively augmented this effect, i.e., the cytotoxic effect of granulocytes and monocytes on mycoplasma-coated target cells. So, I believe that mycoplasma contamination would seriously interfere with the specificity of lymphocyte-mediated reactions.

Herberman: Dr Bean, have you compared "dirty" versus "clean" lines?

Bean: No, not since we began collaborating with Dr. Fogh. Our wish is now to see if mycoplasma does influence the test. Because we are using an isotope assay, we are a little worried about having mycoplasma in our cultures. It is known that the uptake of tritiated thymidine, RNA, and protein precursors will be altered by the presence of mycoplasma.[22]

McKhann: Is the danger of mycoplasma significantly less in short-term cultures than in long ones? That might be a strong argument, for it seems pretty hard to keep it out.

Herberman: Where does the mycoplasma come from? Some people think it is from the tumors themselves; others say from the FBS.

Koldovsky: In a paper at an American Society of Microbiology meeting, it was stated that long-term cultures have up to 90% of mycoplasma and short-term cultures have $<30\%$; it depended very much on whether or not FCS was used for cultivation or not. The FCS had a much higher incidence of mycoplasma. Maybe there is some sort of mycoplasma contamination in FCS.

J. Holland: A number of years ago Halkolay devised a way to keep them out. Horwitz, Herwitz, Halkolay, and I described the high activity of nuclease cytophosphorylase in mycoplasm. And, ever since, Halkolay has grown cells with methotrexate and hypoxanthine, glycine, and thymidine reversal. In the presence of mycoplasma contamination, the thymidine gets catabolized, the thymine in the cells dies, so that it's a fail-safe kind of shut-off mechanism. I think that it is of considerable importance because it does influence the integrity of thymidine for uptake, and not only might it be taken up but it might not be taken up with the nuclease cytophosphorylase activity which is enough to metabolize the thymine.

E. Plata: The problem of mycoplasma contamination literally exploded about a year ago. Commercial lots of bovine serum (100 and 200 ml) were concentrated, and the whole samples were tested for growth of mycoplasma. Most commercial lots were contaminated when they were tested. A circular on the matter was immediately issued by Dr. Hayflick to all concerned. In our experience, FBS was the major source of contamination. Commercial suppliers now can provide certified mycoplasma-free serum. They currently provide us with test results for mycoplasma obtained from 100–200 ml samples of serum lots tested by Dr. Hayflick's facility and one other laboratory. We tested an additional 300-ml sample, of which we sent a subsample to Dr. Hayflick's reference laboratory. We selected for use, roughly, 1 of 12,015 lots tested. The chosen lots meet stringent criteria for absence of mycoplasma and suitability for the particular cell cultures we are using. Suitability is judged on the lack of toxicity at 20–30% serum concentration in medium, plating efficiency, maintenance of morphology, etc.

Herberman: Since Dr. Hayflick is performing his cultures with a limited volume of media, what are the chances of there being mycoplasma contamination in some of the lines that he has called free of contamination?

Cannon: We have found discrepancies between tests for mycoplasma when the same cell lines were repeatedly tested within 1–2 months. We believe that we need to have other laboratories check for mycoplasma contamination or do it ourselves.

Levy: Even those laboratories that concentrate samples of a serum lot before performing their mycoplasma screen are actually culturing only 1/100 of the total volume of the lot. I wonder whether filtration of the

[21] Sinkovics J, Gyorberg F, Cabiness J, et al: Effect of mycoplasma contamination of target cells on the in vitro monitoring of cell- and antibody-mediated immune reactions of patients to cultured tumor cells. Seventy-Second Ann Meet Am Soc Microbiol. Abstracts, 1972.

[22] Perez A, Kim J, Gelbard A, et al: Altered incorporation of nucleic acid precursors by mycoplasma-infected mammalian cells in culture. Exp Cell Res 70:301–310, 1972.

serum through 0.10μ instead of 0.22μ membranes would reduce significantly the mycoplasma contamination. I received from Gibco a quotation of $9.00 per 100-ml bottle for so-filtered FCS, but have not yet used any.

Bean: Maybe I should pass on the helpful information that Dr. Fogh supplied to us. From the early days in his laboratory, when he received his serum and media from commercial companies, he immediately filtered it, set it at 4° C, and waited until the bacteriologic testing was finished on the filtered samples before he used it. He felt in retrospect that this is the reason he has never had any mycoplasma contamination. He subsequently found out that storage at 4° C is a most unstable condition for mycoplasma. We have adopted his preparation method. Now, when we get serum in the laboratory, we filter it immediately and store it for 14 days at 4° C before use. Since starting this procedure, we have had no mycoplasma contamination. We believe that it may be the FCS that was the source. We pass the serum through 1.2, 0.45 and 0.22μ filters in series. Dr. Fogh believes that, while filtration will not completely eliminate mycoplasma, it significantly reduces the number of infectious organisms per ml.

Levy: Does Dr. Fogh have any information on the use of filters with pore sizes $<0.22\mu$? Because of variation in the pore size in any one filter and in the size of the mycoplasma organism itself, 0.22μ filtration does not remove all the viable mycoplasma.

Bean: I don't have the exact data, but he has set up samples of various known infectious units of mycoplasma, done filtrations, and demonstrated a reduced count as the result of filtration. The smallest filter was 0.22μ.

Sinkovics: I wonder about heating sera at 56° C for 1 hour. If the bottle is really submerged, would any mycoplasma manage to survive?

Herberman: Is there any information on whether mycoplasma could withstand such temperatures?

Levy: We heat-inactivate all our sera and still find occasional mycoplasma contamination, but we have never studied the effect of heat on known positive sera. Our discussion so far has centered on serum, but bovine trypsin is another potential source of mycoplasma contamination.

Oldham: If no contamination with mycoplasma has occurred in the past several years in Dr. Bean's and Dr. Fogh's laboratory using a 0.22μ filter and a 14-day, 4° C incubation period, this would seem a very effective method for all of our laboratories to use.

T. Aoki: First, malignant tissues used for tests usually contain many infiltrated lymphocytes that may act as immunologically competent cells. Secondly, this mixture of lymphocytes may cause antigenic modulation or antigen selection. In other words, infiltrated lymphocytes may destroy highly antigenic malignant cells. In such a case, only poorly antigenic cells would remain in the surviving tissue culture. We should be careful about these 2 points.

Herberman: Before changing topics, we should consider the problem of tissue-culture control cells. What is the general feeling about the suitability of skin fibroblasts, even from the same patient from whom the tumor line was obtained, as a control? The objection raised is that, if one gets a negative reaction from the fibroblasts of the patient, this reaction could be due to a variety of things: 1) It's a very tough cell to lyse and a negative reaction may not mean lack of antigen, and 2) fibroblasts are certainly not derived from the same tissue as are most of the tumors studied. Would someone like to comment and perhaps suggest an alternative control that might be used?

Golub: If the control culture is being used and there is concern that it may be resistant to immunologic attack by lymphocytes, one can test for this and determine whether the line is difficult to lyse by using artificially stimulated lymphocytes. Either PHA-stimulated lymphocytes or lymphocytes from MLC's will serve as effector cells and can be used to determine the sensitivity of the target cell to strong immunologic attack. If they are resistant to that, then one can discard them as a useful control.

Herberman: On the other hand, if they are sensitive to that type of cytotoxic effect, will they necessarily be sensitive to weaker forms of attack?

Golub: Not necessarily.

Levy: The choice of proper tissue controls is a difficult task. Ideally, we want a cell identical to the tumor target cell in every way except for the absence of tumor antigens. Please let me exclaim, however, that any normal tissue control is better than none at all! Almost all of our glioma studies are controlled with autologous normal glial cells. But, in 18 instances, we had both normal glial cells and skin fibroblasts as well as tumor cells from the same patients. Ten of these eighteen patients had lymphocytes able to produce 20–30% inhibition of normal autologous cells compared to 60–90% inhibition of their tumor cells. Percent inhibition did not differ between fibroblasts and glial cells, which suggests that fibroblasts were at least as easily lysed as glial cells. We also looked at the effect of various anti-HL-A isoantisera and complement on fibroblasts, melanoma, glioma, and normal glial cells. We found that, while all these cells were affected less than were lymphocytes, each of the 4 nonlymphoid cell types did not differ.

Mavligit: Fibroblasts have HL-A antigens on their surface. However, in MLC they stimulate very poorly.[23] It is probably the nature of the fibroblasts themselves which prevents them from being able to stimulate blastogenesis in allogeneic lymphocytes. If one assumes that immunologic killing is preceded by immune recognition of foreign antigen, conceivably fibroblasts may be the wrong control for cytotoxicity assay. Normal tissues, from kidney, adrenal gland, and other cellular organs, in our hands slightly stimulate blastogenesis among autologous lymphocytes and considerably stimulate blastogenesis among allogeneic lymphocytes.

[23] Schellekens P, Eijsvoogel V: Lymphocyte transformation in vitro. III. Mechanism of stimulation in the mixed lymphocyte culture. Clin Exp Immunol 7:229–239, 1970.

Levy: First of all, Dr. W. Smith and I have seen a blastogenic response of lymphocytes to autologous glioma cells. Regarding the nonstimulatory nature of fibroblasts in the MLC, there are probably factors other than HL-A incompatibility that are important. For instance, fibroblasts may not produce the gene product of the hypothetical *MLR* locus. In your studies with various tumor cell suspensions, normal lymphoid cells that almost always contaminate primary tumor-cell suspensions possibly could induce a blastogenic response in allogeneic cells.

Oldham: Dr. Levy, what percentage of the time do you see "autoimmune" phenomena in your tests? Either toward normal glia cells or toward normal glia cells and normal fibroblasts?

Levy: About half of our glioma patients have lymphocytes that produce 20–30% inhibition of autologous normal target cells, in contrast to their 50–95% inhibition of tumor cells. We have not seen reactivity against normal fibroblasts in our melanoma patients, however.

Oldham: You don't think that half of these patients have autoimmune disease?

Levy: I don't know. By in vitro analysis, they show an autoimmune process. Whether this is clinically significant, I cannot say, but would add that the cancer literature is replete with discussion of the role of autoimmunity in the pathogenesis of the so-called remote effects of neoplasia.

Santos: I'm sorry, but I'd like to open that wound again. You say that it probably means autoimmunity. One of the reasons some of us, at least, are looking at these tests is to make some correlations with what might be occurring clinically so that we can say that this really means something—that it is not an artifact of the laboratory. Therefore, if you're getting this reactivity to normal skin fibroblasts, I'd say that that is interesting in itself and should be followed-up as to what it means. Otherwise, you cannot take the response of the tumor and correlate it to something because it's hardly fair. You're pushing something into a tight shoe to fit a prejudice.

Levy: Autoimmunity is probably a misnomer for the reactivity against normal target cells, since both autologous and allogeneic target cells are equally affected. The significant fact is that we have looked at normal tissue controls and are able to say that the reactivity against specific tumor cells is greater than any effect on appropriate normal cells. The formula with which we calculate our data clearly takes into account any effects on the normal target cells. I don't think anyone can speak of tumor-specific reactivity unless he can show a clear difference between the effects of immune cells or serum on neoplastic and on autologous normal target cells.

Santos: When you block a reaction against the tumor in a patient who also reacts to normal fibroblasts, do you also block the reaction to the fibroblasts or have you done enough of those to know?

Levy: No, the blocking sera have little or no effect on the antifibroblast activity. Absorption of blocking sera with tumor cells, but not with normal cells, does reduce the activity of the sera.

Herberman: Do you have a way of differentiating whether the cytotoxic effects on tumor cells versus control cells are just quantitatively different reactivity against the same antigen, or whether the reactions are against more than one type of antigen?

Levy: We are currently doing lymphocyte absorptions on monolayer cultures of normal and neoplastic cells. These studies should help us answer the very relevant question you pose.

Sigel: About those absorption attempts, Dr. Levy, was the suicide experiment done by the techniques of Bach and others? Essentially, you could try to eliminate subpopulations of lymphocytes active against histocompatibility antigens and, hopefully, retain activity against tumor-specific antigen.

Levy: Such experiments are predicated on the mitotic response of the immune cell and may not be relevant to the cytotoxic lymphocytes in our system.

E. Klein: Using 3 glia and 2 glioma lines as targets, lymphocytes from a number of individuals show cytotoxicity, some possibly due to pregnancies. The 3 glia lines are regularly less sensitive even with these lymphocytes, compared to the glioma cells (and also an osteosarcoma line).

Herberman: Has anyone obtained lymphocytes from people who have been deliberately immunized against particular HL-A antigens, as by blood transfusion or by pregnancy, and tested against fibroblasts bearing those HL-A antigens?

Levy: We have done several experiments of this type both with transplant recipients and volunteers immunized to raise anti-HL-A isoantisera. We do find cell-mediated reactivity against appropriate fibroblasts.

Herberman: Can you demonstrate that there is a high degree of reactivity, in a woman who has been pregnant or in someone who has had a blood transfusion, against the appropriate fibroblasts?

Levy: We have not studied transfusion recipients or parous women, but see little qualitative difference between immunization by transfusion and by intradermal lymphocyte injections as are used in raising anti-HL-A sera.

E. Klein: We had lymphocyte donors with transfusions or 2–3 pregnancies. Many of these donors' lymphocytes lacked cytotoxic activity against the glia lines.

Oldham: I'd like to comment about the types of tissue-culture tumor lines that one can use as reference material, which is not in reference to the fibroblasts. These are epithelial cell lines derived from primary tumors. Table 2 shows the same individual tested 3 times against these 6 cell lines; his reactivity stays relatively stable from day to day. Other normal individuals may demonstrate a level of reactivity that is of the same order of magnitude or they may have levels of activity which are much lower and their reactivity is also reproducible from day to day. The point is that normal controls may have different levels of reactivity against these cell lines. Also,

it appears that certain cell lines are less lysable than others. Line 373 is a good example of this and we've tested now over 40 normal controls against these 6 cell lines at one time or another. The lysability of HT39, G11, and 2563 will be high, BT20 and F-265 will be low, and 373 will always be very low. Obviously then, your controls could be low reactors in terms of the target cells or low or high reactors in terms of the attacker cells. So the problem of what is a good control from both the lymphocyte and target cell point of view is a very difficult one at present.

TABLE 2.—*Reproducibility of reactivity of the lymphocytes of a normal donor, W.B., against several cell lines*

	Target cells*					
Experiment	F-265	BT20	HT39	G11	2563	373
1	12	33	31	50	51	12
2	10	33	29	44	37	2
3	13	33	22	47	55	7
Mean ± SE	12±1	33±0	27±3	47±2	47±5	7±3

*Attacker/target cell ratio 100.1; results expressed as total percent lysis above media control.

McKhann: You said that lymphocytes low against one cell line are low against the other clear across the board, yet at the bottom of the table you have ranges from 55 to 47 at the top down to 7.

Oldham: No, I said that the person shows a reproducible activity against the same cell line from day to day against the same cell lines. He may be low against 373 and high against G11 and, if he is tested the next day, he would still be low against 373 and high against G11. Another person may be low against G11 and low against 373, and he will react at the same level the next day.

Golub: That may not represent differences in sensitivity to lysis by lymphocytes but instead may represent different distributions of antigens. For example, the 373 cell line may have a very uncommon antigen.

Oldham: Yes, that is true.

McKhann: What are the target cells?

Oldham: Line 373 is derived from a colon carcinoma and is CEA positive; 2563 is a lung adenocarcinoma line; the next 3 lines, G11, HT39, and BT20 are derived from breast tumors; and F-265 is a lymphoid line derived from normal peripheral blood. I am not making any statement about the specificity of the antigens on these cell lines, but different cell lines can look different in terms of their relative lysability and also different individuals can be very different against the same cell lines as to their ability to lyse.

Herberman: This emphasizes the importance of a checkerboard type of test for specificity. The size of the checkerboard used and the cells included in the test can make a big difference. If one is putting in just one specificity control line, for instance, a line like 373 could be selected which would give perfect specificity.

Oldham: I agree! If I "pick" my target cell line and if I "pick" my donors in advance, I can have good specificity every time the assay is done.

Levy: Dr. Oldham's results may illustrate a disadvantage of using long-term lines as target cells. I showed data earlier suggesting that some cells, after several passages, lose their sensitivity to immune lymphocytes. Using our early-passage target cells, we see little variation from culture to culture in reactivity to a single lymphocyte donor.

Oldham: I find that very surprising because the Hellströms do not report their results that way at all. They use some short-term tissue-culture lines, and the lymphocyte cytotoxicity values for their normal controls are quite variable from one cell line to another.

Levy: I was referring to patients' lymphocytes.

Herberman: Has anybody else looked at this question of variations in sensitivity to lysis among different cell lines?

Takasugi: Yes, I think you find variability in both. But, in general, the short-term lines are less easily lysed than the long-term lines. Long-term lines usually show a very high target-cell reduction.

McKhann: We have to resolve a problem: What are to be the control cells for the patients' lymphocytes and the patients' tumors. That's the numerator of the factor of how you are going to get there and somewhere along the line you have to have a denominator. It seems to me that we would really have a choice of having the patients' lymphocytes against the patients' fibroblasts as being the basic control. Or you can have allogeneic lymphocytes and the patients' tumors as a baseline control. You can either have a tumor alone as the baseline control or have the patients' lymphocytes or some other allogeneic tumor, but not of the same kind. What is the best control? It is possible to do all these things, but you can only pick one to be the real control.

Herberman: That is an important question, which we will get to a little later. At this point, let's talk about preparation of the attacker cells. There have been some comments on differences in results related to the method of preparation of the lymphocytes, especially a contrast between Ficoll-Hypaque separation and other methods. Would anyone like to speak on this point?

O'Toole: In agreement with Dr. Bean's results, we observed that lymphocytes prepared by Ficoll-Isopaque gave reduced target cell survival as compared with the same donor's lymphocytes purified by nylon wool–Tris–ammonium chloride. The Ficoll-purified lymphocytes appear to adhere more tightly to the target cells in the microplate test.

Herberman: Is it clear in your studies which factors are most important? Is it defibrination versus heparin, or the Ficoll versus nylon, or what?

O'Toole: I have only compared Ficoll with nylon wool–Tris–ammonium chloride using defibrinated blood. The preparations by both methods were equally pure, >98% lymphocytes.

Gutterman: Do they stick more after you heparinize them or defibrinate them?

O'Toole: I can't comment on that since we only used defibrination.

W. Churchill: Have you succeeded in using Ficoll preparations which normally have about 20% monocytes in them? Have you gotten rid of the monocyte fraction and then looked at it?

O'Toole: Yes, we tried Ficoll plus nylon wool and still got lower survival than with lymphocytes prepared by nylon wool–Tris–ammonium chloride. The monocytes do not seem to cause this lower survival rate, since they are removed on the nylon wool.

Sinkovics: Dr. K. Tebbi applied a little trick to get rid of the monocytes in the Ficoll-Hypaque preparation by incubating the buffy-coat leukocytes, before doing anything else, with iron dextran or with colloidal iron, and then centrifuging the buffy-coat leukocytes in the Ficoll-Hypaque gradient. All the iron-laden phagocytic cells which are pratically 100% granulocytes and/or monocytes go down to the sediment. The intermediate layer would be practically pure lymphocytes. This pretreatment with iron dextran seems to produce a pure lymphocyte suspension with the Ficoll-Hypaque technique (*see* Dr. Sinkovics' paper).

Oldham: I would like to show 2 tables with some data about lymphocyte purification and the activity of these preparations in vitro. In table 3, the normal laboratory personnel are in the top group, and you can see that this reactivity is higher than that of normal nonlaboratory personnel who were more closely age-matched to the cancer patients. The first group consisted of 15 normal volunteers (age 20–35 yr). We took the 3 normals out of that group who were the highest reactors against most of the cell lines. We tried to see whether a change in the method of lymphocyte purification made a difference in the cytotoxicity levels (table 4). The common denominator in this table is that a portion of the blood from each patient was placed on Ficoll-Hypaque gradients and the lymphocytes were obtained. So you have a value of cytotoxicity for the media control and then you have a value of cytotoxicity for lymphocytes prepared by Ficoll-Hypaque gradient. So the numbers that you see in the table are plus and minus values for the same individual on the same day as compared to Ficoll-Hypaque lymphocytes. Defibrination plus Ficoll gave all plus values across, and that means that there was a small increase in the cytotoxicity of these normal control lymphocytes when the blood was defibrinated and then placed on Ficoll-Hypaque gradients as opposed to when it was prepared on Ficoll without defibrination. The second line is defibrination plus nylon plus Ficoll, and the standard again is Ficoll alone. These bloods were defibrinated and passed through a nylon column in the standard manner and then the effluent cells were placed on a Ficoll-Hypaque gradient. There was no consistent trend one way or the other in this group as compared to Ficoll alone. The interesting part is in the last group. The standard again was Ficoll alone; the other half of the blood specimen from these same 3 individuals was defibrinated, incubated with plasma gel, and then passed through a nylon column. This was the only group where the lymphocytes were not in contact with Ficoll-Hypaque. As you see, there was a strikingly consistent decrease in the mean cytotoxicity levels for the 3 normal patients against each of the 6 cell lines. And as I alluded to in my talk, I think this is at least part of the reason why our normals were high. Why the young laboratory people seem to be high, whereas the age-matched older controls who do not work in a laboratory are lower, is unclear at this time. We haven't checked the latter group to see if we can also decrease their reactivity by not using Ficoll-Hypaque gradients, and we haven't checked the lymphocytes from cancer patients to see if we can increase the specificity of our reactions by a different method of preparation as yet.

Santos: Have you checked what this does to someone whom you think is specific?

Oldham: We have not yet checked cancer patients in this manner. We will do experiments in which the blood will be split into 2 portions and do the same type of experiments with control and cancer patients to check specificities when lymphocytes are purified differently. This may be the reason the data I already presented had a lot of nonspecific problems. It could also be that lymphocytes prepared by means other than Ficoll-Hypaque may indeed have lower cytotoxicities but no increased specificity.

Levy: With Ficoll plus nylon, did you see a difference between heparinization and defibrination?

Oldham: We did not use heparin plus plasmagel plus nylon.

TABLE 3.—*Summary of reactivity of normal controls and of cancer patients against several human cell lines*

Attacker cells*	Target cells†					
	F-265	BT20	HT39	G11	2563	373
Normal laboratory personnel (age 20–35 yr)	10	16	27	29	27	11
Normal nonlaboratory personnel (age 45–60 yr)	12	8	11	34	20	2
Cancer patients against cell line:						
From similar tumor		9	12	18	19	5
From dissimilar tumor	5	5	12	15	14	12

*At least 10 individuals in each group.

†Results expressed as means cytotoxicity above media control.
Results of several experiments have been combined.

TABLE 4.—*Relationship of cytotoxicity to the method of lymphocyte preparation*

Effector	Target cells†					
Preparation method*	F-265	BT20	HT39	G11	2563	373
Defibrination + Ficoll	+ 2	+ 6	− 3	+ 5	+ 5	+ 5
Defibrination + nylon + Ficoll	+ 7	0	0	+ 5	− 6	0
Defibrination + plasmagel + nylon	− 6	−16	−12	−16	−18	− 3

*Each category represents lymphocytes from 3 normal individuals.
†Each number is the mean change in percent cytotoxicity compared to that of lymphocytes isolated by Ficoll alone.

Bean: In answer to that question, I showed a slide during my talk which showed a cytotoxicity test using lymphocytes from preselected donor's—a normal "killer," a tumor-specific killer, and a normal. We repeated that experiment 3 times and got the same results each time. The second 2 times we did it, it was with blood donors whom we had not previously tested. We found that, with the Ficoll-Hypaque, the specific cytotoxicity decreased as compared with the gelatin preparation. On the other hand, the background cytotoxicity of the normal tended to go away with the gelatin preparation.

Levy: Since March, we have been using Ficoll-Hypaque lymphocytes in the ^{125}iododeoxyuridine (^{125}IUDR) assay. I noticed diminished inhibition but ascribed it to the fact that we were seeing only cytotoxicity as opposed to both cytotoxicity and proliferation inhibition seen with visual counting methods. Perhaps the answer rather lies in our switch from nylon columns to gradients as the lymphocyte-extraction procedure.

Oldham: You mean your reactivities fell when you used the Ficoll-Hypaque? We got the opposite result.

Levy: Yes, but remember that our calculated percent inhibition is based on a comparison of experimental to control lymphocyte effects on the same target cells. Anything that increases the effect of the control lymphocytes will diminish our calculated percent inhibition. We have not used nylon after Ficoll-Hypaque but have incubated our gradient preparations in plastic culture flasks for 15–30 minutes. This simple step eliminated all the monocytes and about half the lymphocytes, but it did little to remove polys that had contaminated the gradient preparations. Would someone comment on the differences between gelatin and plasmagel as erythrocyte-sedimenting agents?

McKhann: I've heard, in contrast to what Dr. Levy has said, that the Hellströms have switched back to using Ficoll-Hypaque in spite of what everyone has said here today and I really don't know why.

Herberman: Another question to be considered is the effects caused by granulocytes. Dr. Takasugi, do you have some comments on that?

Takasugi: We took purified lymphocytes and purified granulocytes from the same individuals and made mixtures from 100% lymphocytes to 100% granulocytes. We found that the percent killing was almost correlated with the amount of granulocytes present as the effector cells.

Herberman: Another factor to be considered is the method used to eliminate or reduce the number of erythrocytes. Some workers use either brief hypotonic lysis with distilled water or lysis with an ammonium chloride Tris solution. Is this harmful to the immunologic activity of lymphocytes?

Mavligit: As far as stimulation with PHA is concerned, we found no ill effect on lymphocyte function following treatment with Tris-buffer ammonium chloride (ACT).

McCoy: In the ^{51}Cr lymphocyte cytotoxicity assay, the shocking of lymphocytes with ACT does have detrimental effects. We have not tried distilled water. ACT-shocked target cells, however, seemed to work quite nicely.

O'Toole: Specific cytotoxicity was not reduced after treatment of lymphocytes with ACT. Removal of erythrocytes makes the microplate test much easier to count.

Levy: When we started doing microcytotoxicity tests, we were afraid of the effect of ACT on the lymphocyte activity and tried some lymphocyte preparations that had been sedimented in plasmagel and passed through a nylon column but had not been shocked. These preparations had 3–5 red blood cell/lymphocyte and produced unacceptably high, "nonspecific" killing. Since then we have routinely used ACT and have obtained good results.

Bean: Could we ask Dr. Takasugi about his original publication on the microcytotoxicity test? The presence of red blood cells that apparently inhibited the cytotoxic reaction was mentioned. Could you comment on that now and do you still feel the same way?

Takasugi: We took a suspension of cells containing a large number of red blood cells as well as lymphocytes. We titrated the suspension on target cells and found that, at the higher concentrations, there was less killing than strong cell destruction and at the lower concentrations this faded out. At high effector cell/target cell ratios, you have the most number of red blood cells in the suspension. We believed that this was interfering with the cellular cytotoxicity. It really should be checked out in greater detail.

Levy: Does anyone have information on the best temperature to keep human lymphocytes before their addition to the target cells? Because of Dr. B. Amos' observation that, for the MLC, human lymphocytes are best kept at room temperature to 37° C, we have always done likewise with our lymphocytes. Recently I've heard some people say that human, like mouse, lymphocytes are best kept at 4° C until they are added to the test system.

McCoy: We have done a study in which we held normal lymphocytes in media with FBS for 24, 48, and 72 hours

at either 4° C or 37° C after processing. These preparations were then tested against ^{51}Cr-labeled F-265 tissue-culture cells. We found that 4° C storage was markedly better than 37° C. The lymphocytes stored at 37° C lost their reactivity in 24 hours, whereas those stored at 4° C for 24–48 hours were still quite reactive in lymphocyte cytotoxicity against F-265 cells.

Levy: I am concerned about the 2–4 hours during which the lymphocytes are being processed. Have you compared 4° C and 37° C for briefer periods? Also you are looking at the activity of normal cells against a cultured line. Has anybody done similar experiments in a specifically immune system?

Golub: I haven't looked at it in terms of preparation of lymphocytes, but by necessity we had to use 24-hour-old lymphocytes which were transported from Nairobi. They were shipped at 4° C and maintained reactivity in response to autologous cells in stimulation tests. Unfortunately, we could not compare them to their reactivity in Nairobi, but their reactivity was good in Stockholm.

Oldham: There's a difference, depending on which test you use. I think Dr. McCoy has found that he can store the lymphocytes overnight at 4° C and he sees a small decrement, if any, in the cytotoxicities using the ^{51}Cr assay. With immune lymphocytes in the rat system in the 48-hour ^{125}IUDR assay, there is a definite decrement after storage at 4° C for 24 hours.

Levy: Is any of the storage effect due to selective reduction in B-cells, since these cells reportedly have a very short in vitro lifespan?

Herberman: A further question that might be asked at this point is: Has anyone had any experience with freezing their attacking lymphocytes, bringing them out, and showing they still have cytotoxic effects?

O'Toole: Yes, they are equally as effective after freezing as they were before freezing, provided purified lymphocyte preparations were frozen.

Levy: We find the opposite. We've gotten positive reactions with fresh cells but nothing with frozen-stored lymphocytes.

O'Toole: Were they viable?

Levy: Yes, they were viable and did work in the MLC.

Herberman: Were they frozen in dimethyl sulfoxide (DMSO)?

Levy: Yes, in our medium with 20% FCS and 10% DMSO, with a freezing rate of 1°/minute.

Herberman: How did you freeze your cells, Dr. O'Toole?

O'Toole: With 10% DMSO and 10% human AB+ serum.

McCoy: Our results agree with those of Dr. Levy. We found very low reactivity as Dr. Levy has stated. In fact, we are attempting to use, as one of our baseline negative controls, frozen attacker lymphocytes. How many different specimens have you tested, Dr. O'Toole?

O'Toole: Very few. I think we've tested 2 known positives this way after freezing.

McCoy: We have had one frozen sample where we thawed out the cells and had some reactivity, but in general, freezing the attacker cells destroys most of the reactivity.

O'Toole: Did you test the frozen lymphocytes for reactivity to PHA?

McCoy: No, only with ^{51}Cr-release lymphocyte cytotoxicity tests.

O'Toole: We tested the PHA in addition to cytotoxicity; the frozen cells responded well to PHA.

Golub: Dr. McCoy, what has been your experience with the storage of cells? Have you determined whether it is better to store the prepared lymphocytes or the whole blood? I am sure that all of us get situations where the sample comes in when everybody is walking out of the door.

McCoy: Yes, we frequently have these situations. One of the problems we have had is getting good Ficoll-Hypaque separation of whole blood, especially if it has been flown in from a collection source and is 12–24 hours old. We frequently have to discard the specimen if the separation is poor.

Koldovsky: For HL-A typing, the so-called Terasaki bag is used. Cells remain in these bags for 7 days and are good. When we use the bags for cytotoxicity tests and go beyond 3 days, the cells lose their cytotoxic activity. For the first 3 days we can use the cells for cell-mediated cytotoxicity. The cells are kept at 4° C.

Herberman: A last question about the attacker cells: Does anyone have any experience with attacker cells such as lymph node or spleen cells?

Sinkovics: Dr. P. DiSaia was working with us. He used ovarian and squamous carcinomas of the uterine cervix cells and found that patients yielded cytotoxic lymphocytes from the peripheral blood, but not from lymph nodes removed from the abdomen. Lymph node suspensions were practically devoid of cytotoxic reactions, whereas the same patient at the same time had circulating lymphocytes with good cytotoxic reactivity. Dr. DiSaia is publishing these results this year.[24,25]

Golub: We've reported the opposite results in the few cases in which we used lymph nodes draining tumor sites from patients with Burkitt's lymphoma.[26] Of course, there may be differences with different diseases, as Burkitt's lymphoma very seldom metastasizes to the lymph nodes. Has anybody been able to separate out lymphocytes from tumor material?

Levy: Dr. B. Hattler has used Ficoll-Hypaque to extract lymphocytes from rejecting dog renal allografts and found these lymphocytes very reactive in an MLC. We

[24] DiSaia P, Rutledge F, Smith J, et al: Cell-mediated immune reaction to two gynecologic malignant tumors. Cancer 28:1129–1137, 1971.

[25] DiSaia P, Rutledge F, Smith J: Cell-mediated immunity to human malignant cells: A brief review and further studies with two gynecologic tumors. Am J Obst Gynecol. In press.

[26] Hewetson JF, Golub SH, Klein G, et al: Cellular reactions against Burkitt's lymphoma cells. I. Colony inhibition with effector cells from patients with Burkitt's lymphoma. Int J Cancer 10:142–149, 1972.

have used his method to extract lymphocytes from brain tumors but have not tested the cells.

Sinkovics: In answer to Dr. Golub's question, we have several established lymphocytic cell lines, among them long-term suspension cultures derived from the biopsy of an angiosarcoma. It was not the sarcoma cells but the lymphocytes that grew. Another lymphoid cell line from a breast carcinoma and one from a malignant melanoma were also established. These lymphoid cell lines are nonspecifically cytotoxic on various target cells; i.e., no specificity to angiosarcoma, breast carcinoma, or malignant melanoma could be established when these lymphoid cell lines were tested against the corresponding tumors *(see* Dr. Sinkovics' paper).

Koldovsky: Many years ago the tumor from biopsy was described, mainly its periphery, as capable of being infiltrated by lymphocytes or eosinophils. Such infiltration is a good prognostic sign.[27,28] It seems that these cells are not immunologically paralyzed but can react against the tumor cells.

Herberman: What differences have been observed when the target cells were suspended in FBS versus human serum, or when cells were grown in FBS versus human serum? With cells in FBS, some positive reactions might be due to immune reactivity against the fetal bovine components.

Levy: We've never grown the cells themselves in human serum. We've received them in FBS and then washed them before doing the tests with medium containing human AB serum. From then on, the test was done in human AB serum, but we've never actually grown the cells themselves in human AB serum.

Takasugi: Dr. Levy, did you repeat the same test, one with human serum and the other with FBS?

Levy: Only once have we directly compared human AB serum and FBS. We found much greater "nonspecific" killing with FBS.

Bean: I'd like to show a table of an actual assay that was run in 10% human AB+ serum and in 10% FCS in parallel (table 5). As you can see, we had 34 cells/well at the start of incubation (time of addition of lymphocytes). In both sera, cells divided during the assay period but not to the same extent. You can evaluate these results in a number of ways. But the same target cells and lymphocytes give different results in different serum. This also illustrates the problem of cell division that led us to turn to an isotope. E.M., a melanoma patient, started undergoing regression of all of her skin nodules, and we were lucky enough to get some of her lymphocytes. What we see here is a reverse cytotoxicity. She had statistically more cells remaining in either the 10% FCS or AB+ serum than anyone else. She clearly differs from everybody else. And she is regressing her melanoma. I believe that this stimulation of division may be related to the Ficoll-Hypaque, but it could be a characteristic of the cell line. Each particular cell may react differently. Some may be stimulated to divide on specific contact with lymphocytes and some may be destroyed.

Herberman: This is the only time that you have done this?

TABLE 5.—*Lymphocyte cytotoxicity assay—effect of sera*

Lymphocyte donor*	Number of melanoma target cells remaining with assay in: 10% human AB +	10% fetal calf
None—start of incubation	34± 3	
None—media control	67±11	136±14
P.W., normal	78±20	73±11
C.P., normal	119±17	96±16
E.S., bladder carcinoma	116±15	84±14
O.R., normal	113±16	75±12
A.R., progressive melanoma	125±22	109±20
S.M., regressing melanoma	193±19	152±13

*200,000 lymphocytes (defibrinated. Ficoll-Hypaque, nylon column) incubated 60 hours.

Bean: Yes. Now that we have an isotope system, we will go back and compare sera which should be more valuable than these are.

Golub: I have tried it only twice in the IUDR test, and we apparently got less nonspecific killing with human AB serum than with FCS. We also tried human agamma serum which at least has a theoretical advantage of not containing any potential blocking antibodies that might be present in the pooled AB sera. Human lymphocytes do not proliferate quite as well as in agamma sera as they do in AB sera, but they appear to be able to mediate a cytotoxic response.

McKhann: Does anybody cocultivate the lymph node cells for any specific period, such as 24 hours without any kind of sera and then with media plus serum in it?

Koldovsky: First of all, I don't think it hurts too much if the FCS sticks—even for a few generations on the cells. But on the other hand, if you are using control cells such as skin cells from the same patient, you are cultivating usually in the same FCS. If the FCS is responsible for cytotoxic reactions, your control cells should react too.

Levy: I am not as concerned about FCS's sticking to the target cells as I am about the blastogenic effect of the FCS on normal and sensitized lymphocytes.

Herberman: What about the size of the wells that are used in the test plates? There is certainly a wide range used, from the very small wells that Dr. Takasugi is using up to the size that Dr. Oldham is using. Does this make a big difference in terms of the nonspecific or specific effect, either because of nutritional factors that Dr. Takasugi alluded to in his talk or because of the geometry of the wells? Did anyone perform comparative tests in plates with different size wells?

Bean: Just one theoretical consideration that we would like to clarify. We originally believed that the contact

[27] YOON L: The eosinophil and gastrointestinal carcinoma. Am J Surg 97:195–200, 1959.

[28] BERG J: Inflammation and prognosis in breast cancer: A search for host resistance. Cancer 12:714–720, 1959.

between target cells and effector cells was probably much better in a small plate (Falcon 3034). People tend to refer quite a bit to the effector/target cell ratio, and this may not be as important as the effector/target cell/area ratio. If you are going to use 50 target cells in the 3034 plate wells, you would have to use approximately 1,100 cells in the Falcon 3040 plate to achieve the same kind of density.

Levy: Apropos to Dr. Bean's comments, Dr. G. Berke has been able to manipulate his EL 4 allograft system by rocking and stop-rocking the culture dishes. In a paper now in press in *Science,* he describes experiments in which he stops the cytotoxicity reaction at an intermediate stage merely by stopping the rocker. I have been unable to get much cell mixing by rocking the Falcon Microtest II plates and wonder if this has limited the extent of cytotoxicity and increased the lymphocyte demands of my system.

Herberman: Over what time period does Dr. Berke perform these tests?

Levy: One to four hours and sometimes less.

Herberman: This is certainly true in the Brunner or Canty short-term assays. Rocking is very important; this is an observation that most laboratories have made. In long-term assays of 24–48 hours, this may not be as important.

McKhann: Dr. Clark, of Boston, has emphasized that at least in his work, he had to have larger wells and he had to rock it to get it to work.

Dr. P. Cleveland in my laboratory tried this in a smaller well because we had not been rocking. The surface tension of the wells may preclude any real motion. When we tip the entire microscope slightly, there is no motion in the cells at all.

Levy: What volumes did you use?

McKhann: At least 0.2 ml/well.

Levy: Would it help if the volumes were smaller?

McKhann: It would probably be less because there would be more surface tension.

Bean: I've wondered if perhaps this is why the Hellströms insist that you have to rock for at least half an hour in the absence of FCS. Perhaps the lower viscosity of the fluid without the serum facilitates lymphocyte/target cell contact and that might be why this is so critical. Dr. G. Heppner also believed this was very important in the assay she used in her laboratory. Perhaps Dr. Byrne could comment?

Byrne: We, in fact, do that. But before we had a rocker it used to work. I don't know if we could be as certain of that as you thought, but I do know that the assay was working before we got the rocker and, since we started using it, we really haven't systematically tried to find whether the assay works with or without rocking.

P. Cleveland: Referring to Dr. McKhann's comments about comparing rocking versus nonrocking, we found that, using these microtiter mixers, we can resuspend our lymphocytes quite well as long as we have only 0.1 ml of media in our micro-3040 plates. But, if we put 0.2 ml on the plates it is difficult to suspend the lymphocytes and agitate them; if we put them on Bellco rockers, we will get a motion of the lymphocytes back and forth. In addition, when we compared rocking versus nonrocking and a combination of rocking and agitating, we had better killing when we agitated our cells than when we just let them sit stationary.

Takasugi: We also found that, when we put plates on a rocker, the cells shifted from one side to another and then came to rest with most of the cells on just one side of the well.

Levy: We have not found it necessary to incubate the lymphocytes on the target cells briefly without serum. Routinely, we dilute the lymphocytes in medium containing 10% human AB serum and add them in a volume of 0.2 ml.

Herberman: Does anyone want to comment on what is actually being measured by the different assays? Is one looking only at cytotoxicity with the radiolabeled techniques and measuring cytotoxicity plus growth inhibition and loss of adherence in the microcytotoxicity assay? What about other differences between the assays?

McKhann: In the radiolabel assay, where the label is going to be passed on and divided between all succeeding generations of cells, if there is any growth of cells (but also from killing), the loss of label will be recorded as a loss regardless of what else goes on in culture. And if it's a matter of counting cells, you could conceivably have some cells being killed and some cells dividing, and it would give you no net change. So there is a subtle difference between what is actually being counted 2 or 3 days later after you started the cocultivation.

Levy: Does anyone know what percentage of the target cells are labeled? Specifically, I'm interested in the ^{125}IUDR technique. Also what is the evidence that ^{125}IUDR is not reutilized?

Oldham: I can't answer your first question, but we did some correlations between visual counts and isotope counts. It was my impression that there was approximately one count per cell in the rat system.

Levy: Yes, but that could be a thousand counts in one of a thousand cells.

Oldham: ^{125}IUDR is reutilized at a very low level; perhaps about 2–4% can be reutilized according to several published reports. Both in vivo and in vitro, ^{125}IUDR is reutilized at a very low level, whereas, in comparison, tritiated thymidine is reutilized at a very moderate level.

Levy: Is it correct that, once a cell takes up IUDR, it no longer can undergo mitosis?

Oldham: Yes, that has been published already. We've done the same experiments with the F-265 lymphoid line. When we labeled F-265 cells with IUDR, we found that after 24 hours the same number of cells were there as before. And if you look at your control cultures, the number will not only double but actually increase by a factor of three. So apparently nearly all of the cells incorporate isotope and then they don't replicate after that. But in these assays that's really not the problem: all you're really worried about is keeping the

toxicity to your cells at a low level. For our assay, it may be better that the cells do not replicate because we want to work with the cytotoxiciy and not be concerned with the problems of cell replication during the assay.

D. Yohn: Does any of your iodine appear in your attacker cells after the reaction?

Oldham: No.

Herberman: Let's address ourselves to some of the other variables in an assay. The length of incubation was mentioned. Aside from the ^{51}Cr-release assay, which is often quite short term, most of the other assays are run for 24 and 72 hours. How short can one make an assay of this type to get specific results? Does one get somewhat different results depending on the time that one incubates the lymphocytes? We have data from a Gross leukemia system in rats: Lymphocytes from rats, any time after 40 days following immunization, are unreactive in a direct short-term ^{51}Cr-release assay. However, if we preincubate these lymphocytes at 37° C for 18–24 hours, the lymphocytes are quite cytotoxic for the target cells in the assay. There appears to be an activation of the lymphocytes when they are taken out of the environment of the host.[29] This might explain some of the differences between a short-term and a microcytotoxicity assay.[30] Does anyone want to comment about that?

Golub: One point to remember is that at 48 hours one can begin to detect in vitro sensitization of lymphocytes. Differences in cytotoxicity may represent different rates at which sensitized and nonsensitized cells respond to antigen. We did not use the cytotoxicity assay to do any time studies to define the kinetics of the cytotoxicity as measured in our colony inhibition assay. It could well be that the actual time course required to detect cytotoxicity in the microcytotoxicity assay could be much shorter than what has been reported. I would ask a general question now: How many people have tried to use shorter incubation times with the microcytotoxicity assay? And what results have they gotten? In other words, could one considerably shorten the period of incubation that is being used routinely for the cell-mediated reactions?

Herberman: Dr. Oldham, you had some data on incubation for 24 hours. Does anyone have any data for less than 24 hours with these types of assays?

Levy: We have looked at 12, 18, 24, 36, 40, and 48 hours and find nothing until 24–36 hours, with the peak effect seen at 40 hours.

Herberman: What about the influence of pH? Do the assays have to be done in a CO_2 incubator? Dr. Levy mentioned in his talk that it doesn't seem to make any difference. Does anyone have any information about this?

[29] Ortiz de Landazuri M, Herberman RB: In vitro activation of cellular immune response to Gross virus-induced lymphoma. J Exp Med 136:969–983, 1972.

[30] Wright P, Ortiz de Landazuri M, Herberman RB: Immune response to Gross virus-induced lymphoma: Comparison of two in vitro assays of cell-mediated immunity. J Natl Cancer Inst 50:947–954, 1973.

One of the variations that people have done in the assays has been to add the attackers to the cells while they're still in suspension and then to plate out the combination together. An alternative has been to add the lymphocytes to the monolayer after the cells have been plated. Is there any information about which method is preferable?

Takasugi: We did it both ways. Actually you see the same type of results, except that, if you add the lymphocytes to target cells that are already adhered, the variability is less.

McKhann: We've tried it both ways and found interference with plating of the target cells in the presence of high levels of lymphocytes; i.e., if the target cells have already had a chance to plate, then there's much less variability.

Oldham: Our work confirms what Dr. McKhann just said.

Herberman: Has anyone else tried the method Dr. McKhann mentioned, of removing the nonspecific cells sometime during incubation? If not, one major area that we ought to talk about is the attacker/target cell ratio and whether it is important to test more than one ratio and get a response curve. With the microcytotoxicity assay, a wide range of ratios have been used, from 5:1 up to 5,000:1.

McCoy: Table 6 shows results of the ^{51}Cr-release assay in which the attacker/target cell ratio was varied. In this particular experiment, we held the number of labeled tissue-cultured F-265 lymphoid target cells constant at 5×10^4 and varied the number of attacker cells from 5×10^6 down to 5×10^3, thus giving attacker/target cell ratios of 100:1 down to 0.1:1. The degree of lysis of labeled F-265 cells decreased as the attacker/target cell ratio decreased. The amount of lysis, however, was quite good even at ratios of 20:1 and 10:1. Interestingly, ratios as low as 2:1 and 0.2:1 produced significant lysis of F-265, suggesting that low numbers of lymphocytes, and possibly even less than one lymphocyte, can kill 1 target cell.

TABLE 6.—*Effect of decreasing the attacker/target cell ratio (target cell number remaining constant) in the lymphocyte cytotoxicity*

	^{51}Cr-labeled target F-265 cells (5×10^4)
Attackers	F-265
Autologous	6.0%
Donor lymphocytes	
100/1 (5×10^6)	18.9 (0.9)*
60/1 (3×16^6)	16.9 (1.5)
20/1 (1×10^6)	14.1 (1.3)
10/1 (5×10^5)	9.1 (0.5)
2/1 (1×10^5)	5.1 (0.4)
1/1 (5×10^4)	2.9 (0.5)
0.2/1 (1×10^4)	2.3 (0.4)
0.1/1 (5×10^3)	1.0 (0.9)

*Percent lysis: in parentheses, mean SE.

Oldham: I showed a slide earlier, in which ratios of 10:1 to 100:1 did demonstrate a dose-response curve in

the rat system. Above 100:1 you have a slightly higher cytotoxicity at 200:1, but at 500:1 the curve is completely flat and cytotoxicity level does not get higher with attacker/target ratios above 500:1. With the nonimmune cells, cytotoxicity continues to rise until about 750:1. At this point, there is very little difference between immune and nonimmune lymphocytes.

Levy: Dr. G. Berke has found that, while the killer/target cell ratio was important, if he kept the ratio constant and increased the target cells, he got a greater percentage killed. We have not found this to be true in our system. With our IUDR technique, we routinely use 10:1, 50:1, and 100:1 lymphocyte/target cell ratios and see killing at 50 and 100:1 if we use 1,000 target cells. As we increase the target cell number, keeping the ratio constant, we see less killing until, at 10,000 cells/well, we see no difference between control and experimental samples.

E. Klein: We believe titration is necessary; also it is good to have the low ratios. It is important to perform cell titration; e.g., some cell lines are influenced by the presence of lymphocytes, and they grow better due to a feeder effect. Superimposed on that, there might be a cytotoxicity, which is then revealed by the slope of the curve.

Herberman: Dr. Byrne, you are working with a high attacker/target cell ratio. Would you like to comment?

Byrne: In the microcytotoxicity test we are using lymphocyte/tumor cell ratios of 1,000:1 and 5,000:1 and a visual counting method. With the soft agar method, it is possible to use ratios of 100:1 and 10:1 and get reproducible results.

Herberman: Even with the visual method, it sounds as if there is a lot of differences between some of the groups. Dr. O'Toole and Dr. Klein are sometimes using ratios as low as 100:1 or 50:1.

Byrne: Yes, but they are using different plates.

Herberman: As you go to around ratios below 1,000:1, do you lose the effect?

Byrne: Yes.

Rubin: It seems to me that we are talking about what the optimal ratio is. Everybody is working on different tumors which may have different qualitative and quantitative distributions of antigens. No one yet knows how many specifically immune lymphocytes it takes to kill 1 target cell, and it probably isn't the same for a breast cancer cell as it is for a lymphoid cell. I don't know how far you can go in comparing just the ratio without specifying the tumor system being studied.

Herberman: We should also discuss the methods by which the attacker/target cell ratios are calculated. One might add a counted number of cells to each well but then let these cells proliferate for 24 hours before one adds the lymphocytes. The actual ratio would then be lower than the one calculated. The issue I am raising is a very practical one. Some of the differences between investigators on the ratio used may be more apparent than real. Are the ratios just being calculated differently? Some people might count the number of target cells in the dish when the lymphocytes are added and base the ratio on that. Others are basing the ratio on the number of target cells added to the plates before the 24-hour incubation. Would anyone like to comment?

Koldovsky: Our problem is more complicated by the fact that you don't know what percent of the immune cells you are using are really immune. It can be anything from one-tenth of a percent to 2% in a good situation. You also don't know how many immune cells can kill 1 target cell. Based on experience with adoptive transfer of immunity, a reasonable guess is that it is necessary to use 10–15 immune cells to kill 1 target cell. However, when target cells are producing antibodies, one can look at the plaque formation inhibition, and in such, 1 immune cell can kill 5 target cells.

McKhann: Some tumors have poor plating efficiency and you may have fewer cells after 24 hours than originally and not more.

Herberman: This will give a considerably higher ratio than you calculated.

Takasugi: In the small plate, the counting is very inaccurate. You can count cells to add 500/well, and then when you look at it you only see about 200 or 300. It is much less than what is calculated.

Bean: That's one of the reasons we've switched from using that plate to the larger plate (3040), because we had many plated cells on the side walls in the 3034 plate.

Takasugi: But we would like to stay with it because it is so much easier to count on machines.

Rubin: I don't know if you really want to get into this now, but it is an important issue. It is not a technical question, but ultimately a matter of whether these tests are measuring qualitative or quantitative differences in cellular reactivity. If what you are calling nonspecific killing really is due to the fact that, in a representative population of normal people, only a few have enough of these *specifically* sensitized cells circulating to be detected by the current methods. On the other hand, the patient with a tumor has an amplified clone of such reactive cells. This does bear on what you mean by the ratios and their interpretation of what is specific killing.

Herberman: If these are quantitative assays and if there are dose-response relationships, then one should be able to draw curves and to see whether the slopes of the curves produced by the lymphocytes from the tumor patients are the same or different from those produced by control lymphocytes. With a short-term assay, like ^{51}Cr-release assay, one gets a good dose-response relationship. This is the basis for the inhibition assay Dr. McCoy described. However, can one get straight-line curves that are reproducible with the microcytotoxicity assay?

E. Klein: With our experiments, as the number of lymphocytes increases, the percentage of target cell survival decreases. Some normal individuals show a killing effect with the same dose-response curve as the patients.

Herberman: Perhaps you can use that to argue that you have the same type of effect with the normal

lymphocytes as with the patients. I would now like to discuss the methods used for calculating results. Is it fair to pick a particular normal individual to use as the baseline and to compare the results of the patients to that? Have the groups who are doing this looked at enough normal individuals to know whether there is much variation in reactivity within a normal population. Also, the definition of percent cytotoxicity varies from one group to another. The calculation Dr. Oldham used is quite different from some of the other calculations used. Dr. Oldham, you raised this question; do you want to comment on it?

Oldham: Most of the investigators in the literature have calculated their results in terms of a cytotoxicity index (CI). This usually goes something like:

$$CI = \frac{\text{counts/minutes (cpm) in control wells} - \text{cpm in experimental wells}}{\text{cpm in control wells}} \times 100.$$

This index is related to the number of cells left in the bottom of the wells for the control wells (be it media control or normal control lymphocyte) in comparison with the number remaining in the test group wells. Table 7 is from our ^{125}IUDR assay. The only thing I would point out here is that 2 individuals were both highly cytotoxic for this particular line. The media control here was 53%; consequently, there were very few cpm as well as very few cells remaining in the wells at the end of the assay. If there are a lot of cpm or a lot of cells left in the wells, the calculation of a CI seems to work fine. As the numbers get very small (as shown here), difficulties arise. For instance, we calculate our results as percent cytotoxicity based on the amount of isotope released over the total amount originally in the cells. Using this method, you get a cytotoxicity for the breast patient against G11 (a breast cell line) at 97%, and the normal (JP) against the breast line at 93%. This would not be a significant difference in cytotoxicity by our criteria. Yet if you calculate, with the same numbers, a CI, you will find that the CI is about 75%. The general level of significance (the 0.05 level) in the literature is a CI >25–30%. Many of the CI's so reported are in microcytotoxicity assays when the cell numbers are in this range of 50–100 remaining at the end of a test. So I wonder if the CI with these low numbers is directly related to the cytotoxicity or is it partially a statistical artifact?

Mavligit: If 20 cells, remaining after use of lymphocytes from the patient and 30 cells remaining after use of lymphocytes from the control, give a CI of 33%, then 2 and 3 cells remaining, respectively, should also be considered as a CI of 33%. This is, of course, absurd because there is no difference between 2 and 3 cells remaining or between 6 and 9 cells remaining. Since it is all subject to statistical manipulation, I just wonder if the patient himself should not be his own control in a sequential study with correlation to his clinical status.

Herberman: The point that Dr. Oldham is bringing up is different from that; it's whether one is calculating a CI and calling it percent cytotoxicity—where one is not measuring cells or isotopic counts in the supernatant or the percent of cells being killed, but rather looking only at the fraction remaining in the experimental wells versus the control wells. When there's a high degree of cell loss in both experimental and control wells, looking at both the numerator and the denominator of that would give a much different picture than just looking at the numerator.

Oldham: To call the breast patient positive for immunity and the control negative in the previous table would not seem very reasonable, since they both kill >90% of the target cells; yet with these same numbers, a highly significant CI of 75% is calculated.

Takasugi: I don't think I've ever reported the CI. It was always with a medium control.

Herberman: But, even with the way you are calculating, Dr. Takasugi, it still is quite different from that of Dr. Oldham. You're only looking at the number of cells remaining in the wells in the experimental versus the control. You don't have an actual measure of how many cells have been lost during that period. I'm not sure you can do this with a microcytotoxicity assay.

McKhann: It seems clear to me that you have a lot

TABLE 7.—*Calculation of cytotoxicity*

Lymphocytes			Target cells		
			G11 (cpm)		
Patient	Tumor type	Ratio	Supernatant	Cells	Percent cytotoxicity
D.H	Breast	100/1	2951	49	
			3512	58	97*
			3377	79	
J.P.	Normal adult	100/1	3307	164	
			3503	436	93*
			4271	162	

*Not significantly different levels of cytotoxicity.

$$CI = \frac{\text{Number of cells (cpm) in control group—No. of cells (cpm) in test group}}{\text{Number of cells (cpm) in control group}}.$$

$$CI = \frac{254-62}{254} = 75\%.$$

of cells lost nonspecifically, in both the normal nonimmune lymphoid cell and the media control which you have in the assay.

Bean: I can see both sides of the story here. I can envision problems using the type of calculation when you use a cell line not readily adherent. We all know that some cell lines tend to attach better than others and some cells tend to be released at high density, which is what you have with target cells in the presence of lymphocytes and don't have in the media control.

Oldham: May I add that there is a media control which is down around 50%, as it usually is. G11 is a plastic-adherent cell line which does not come off the plastic easily. Both individuals' lymphocytes are cytotoxic on this occasion. The point of table 7 is not the level of cytotoxicity of the normal control, but rather the relationship between the high-reacting normal individual and the cancer patient and the way you calculate the results. If you look at the numbers in the tables of papers on microcytotoxicity or colony inhibition, the number of cells remaining in the wells at the end of the assay is in the range of 50–100, so, as shown in table 7, this may be introducing a significant artifact.

Martin: I am somewhat skeptical of quantitative interpretations of such assays. No doubt these assays indicate qualitative differences between lymphocytes of tumor-bearing and control subjects. The significance of the degree of difference, however, must await understanding of the in vivo function of the cell type or types reactive in these assays.

Bean: This assay is so difficult that many of us have gotten so far into it technologically that we've lost a little of the perspective. With this kind of assay, if you can take a large number of bladder patients and a large number of controls and show that you have less cells remaining at the end of the assay with bladder cancer patients' lymphocytes, then you have a phenomenon which shows a degree of specificity. Then this phenomenon should be investigated as to what types of mechanisms are involved in it and what the value of this will be for immunotherapy and immunodiagnosis.

Herberman: In an isotopic release method, one can calculate the percent lysis of the total cells added to the wells. However, if in one of these assays, like the ^{51}Cr assay, one just looks at the supernantant counts and does not make a ratio of that over the total number of counts in the cells, results usually called 2–5% lysis above the background may become 30 or 40%. So the method of calculation can magnify or diminish the appearance of the results.

Oldham: I would like to emphasize, in response to Dr. Bean's question, that these are not nonadherent cells contributing cpm's to the supernatant. You can take the supernatant from these test wells, spin it down, and count it. We found, when this was done, there was not a cell button present with high cpm compared to the supernatant. So the cpm in supernatant are derived from *lysed* cells, not from nonadherent cells in an assay.

Herberman: We should also discuss the calculation of results based on the baseline of the normal control lymphocytes. Dr. Byrne, I believe all your calculations are done that way?

Byrne: Lymphocytes from tumor patients and from normal controls are added to wells containing tumor cells. Our calculations are based on the number of cells remaining in the wells after exposure to lymphocytes. The effect of lymphocytes from tumor patients is compared to that of control lymphocytes. The statistical analysis is not done on the percent difference but on the basic figures. We only express a percentage inhibition if there is a statistically significant difference.

Herberman: If one takes a particular normal individual and he has low reactivity, and if one tests the patient and he has higher activity, it looks as though the patient is positive. Yet if one would take that particular patient and 10 normal individuals, perhaps one would see a range of activity from the normal individuals, and the results with the patient might fall within that range. Then, it would be called a negative test.

Byrne: We test 2 normal individuals with each test and I agree that perhaps it would be better to have more. From a practical viewpoint, with the method we are currently using, 4 or 5 simultaneous lymphocyte extractions are the most one technician can manage.

Herberman: Have your normal individuals been selected by any criteria?

Byrne: No. Initially we had believed that certain of our "normal" population showed consistently greater ability to kill melanoma cells than the rest of the normal population. Further testing has not proved this to be so. We are no longer selecting our normal population.

E. Leonard: Since you don't select the melanoma patients, how are you justified in selecting out some of the normal individuals?

Byrne: If there are some which form a distinct subgroup of the normal population, they should be looked at separately. We are trying to get a clinical correlation with the test as we do it. We could go on and try to investigate normal individuals exhaustively. We haven't chosen to do that. We're looking for clinical correlations with this test. My point is that, if one has a test which has clinical correlations, then it has value whether one understands its mechanisms or not.

Leonard: All of your patients have cancer, but are otherwise unselected. If you select all of your normal population for little background activity, you can't fail to find positive results.

Byrne: I think you've missed my point. We are not trying to diagnose cancer. If the test doesn't have a correlation with the *clinical activity* of disease then the test is no good, but if it does, then it may have a clinical value.

Herberman: Dr. Byrne, do you use the very same normal individual every time you do the serial test on a particular patient?

Byrne: No we don't, but we use the same group of people.

Herberman: Do you think that's a good baseline, then?

You're following one person serially and doing multiple tests on that person, but your baseline of comparison may be changing from one time to another.

Cohen: A serial study can only be done with a patient as his own control. If you're going to evaluate how a person is changing from week to week and from month to month, you have to use his own control. If you're basing each test on different controls, you don't know what may be happening.

Byrne: You mean we should freeze down the patient's lymphocytes, reawaken them at a later date, and assume that they would have the same activity as they had when we froze them? Then we would test them against the patient's fresh lymphocytes?

Golub: I think you can use selected normal controls, but then you're obliged to do it in an epidemiologic style. If you preselect your normal controls for a low, nonspecific reactivity and then take a panel of perhaps 10 normal controls that you have screened in this fashion, to be logical about it, you just cannot test your cancer patients, but you must test varied populations and compare them to this one group. Test your specific cancer patients, test your patients with other types of cancer and nonselected normals, and see if there are any differences between each group tested against this one selected population.

Herberman: The point I was making, Dr. Byrne, would not require freezing of cells. You are following over a period of time a patient's activity, which may be constant. If, in each test, you use a different normal control and activity among normal controls varies, this will affect your calculations and give you results indicating a change in the patient's reactivity, whereas the patient has been stable over this period.

Santos: I would like to ask Dr. Byrne two questions. What is the specificity of the cytotoxic activity of his lymphocytes, and if there is antibody specificity, has he thought it might have some significance? For example, has he done other tests to see what happens to the unreactive patient with a particular tumor, to see if transfer factor plays any role? If some of those studies were done, it would help clarify the problem.

Byrne: With regard to the first question, we have not run routinely a tissue control in our assay system; we have, however, used normal lymphocytes as a lymphocyte control.

Santos: My question was about the normal individual who reacts nonspecifically. If there is specificity to it, that is quite as exciting as a patient reacting to his own tumors and it may mean something else.

Byrne: We have reviewed the reactivity of all our normal controls and found that our original assessment, that some had specifically toxic lymphocytes, has not been borne out.

Aoki: How many lymphocytes are required to kill 1 target melanoma cell? In the H-2 system, we did such an experiment to study this problem. We mixed EL 4 target cells with hyperimmune lymphocytes, incubated and fixed them, and made a serial section for the electron microscope. Then we calculated the number of lymphocytes required to kill a single target cell. In some cases, it took only 1 lymphocyte to kill 1 target cell, and in other cases it took 2, 3, or 4. Since you are discussing very broadly here, I don't understand why one has not checked carefully how many lymphocytes are required to kill 1 target cell. Otherwise you cannot solve this problem.

Herberman: With the ^{51}Cr assay, there is evidence that less than 1 attacker cell is necessary to kill 1 target cell, so that there must be multiple killing by individual cells.

Levy: Is this in a syngeneic system or in the allogeneic system as is used by Berke, Wunderlich, and others?

Herberman: Wunderlich, using an allogeneic system, probably has the best data on this. We have some data on a syngeneic Gross rat system which looks like it is in the same direction. *See also* Dr. McCoy's data, table 6.

Takasugi: I finally remembered what I forgot earlier. With about 23% of the cases, we see a feeder effect. In other words, there are more target cells surviving in wells with lymphocytes than the media control. Has Dr. Byrne or Dr. Oldham seen that? How would they handle it in their calculations?

Oldham: We have seen it rarely. If you add F-265 as the attacker against the 6 cell lines previously mentioned, usually the cpm remaining in the target cells are similar to the media controls, but occasionally F-265 seems to protect the target. We have not seen this effect in our assay when lymphocytes from normal individuals or cancer patients were used as attackers.

Takasugi: Does your percent kill become negative?

Oldham: No, it stays about the same as the medium control, but there were some exceptions. The feeder effect that was there was usually only about 5–10%.

Sinkovics: We work with a squamous carcinoma cell line which occasionally phagocytizes and digests lymphocytes and sometimes grows much better in the presence of lymphocytes. We were thinking of growing this cell line in a serum free medium and feeding it with lymphocytes.

Herberman: Before ending the discussion on cytotoxicity assays, we should consider the data and problems associated with blocking factors.

Sinkovics: We have patients who possess some blocking activity in their sera against the cytotoxicity of their lymphocytes, and some of these patients received continuous infusion with cytosine arabinoside (ara-C). We find that the blocking activity of the serum disappears after ara-C infusion. Ara-C is very seldom effective against solid sarcomas; while the blocking factor disappears, the sarcoma continues to grow. A serum drawn from a patient before treatment with ara-C was used to pretreat cultured rhabdomyosarcoma cells and the pretreated cells were exposed to cytotoxic lymphocytes of the patient. There was a cytotoxic effect and there was also a blocking effect. Serum was drawn again 5 days after infusion with ara-C and was used for pretreatment of the rhabdomyosarcoma cells. Then the cells were exposed to cytotoxic lymphocytes; the blocking activity of the serum seemed to disappear. This patient, while

having no blocking activity, continued to have growing tumors and she eventually succumbed. The cytotoxic lymphocytes and the blocking factors appeared to be specific to rhabdomyosarcoma. It is interesting to speculate about these types of studies. If we accept that the blocking factors are complexes of tumor antigens and 7S globulins, we should also surmise that the release of tumor antigens continued because the tumors did not cease growing. On the other hand, ara-C is an immunosuppressant and thus might knock out the other component of blocking complex, the 7S antibody. Therefore blocking activity as such cannot be measured. Very recently Calabresi and his associates showed that in the mouse the most sensitive immunologic faculty affected by treatment with ara-C is 7S antibody production. All the other faculties diminish only after large doses of ara-C.[31]

Levy: From the data you have shown, you can draw no conclusions on the role of blocking antibody in the patient's clinical course because you do not show the effect of ara-C on the patient's cell-mediated antitumor reactivity. If there is no cell-mediated immunity, be it due to ara-C or to blocking activity, you should have the same clinical results; the patient should do poorly. But another interesting point does come out of your data, i.e., the question of whether antigen alone can block. Presumably, these patients treated with ara-C would have the same amount of circulating antigen as untreated patients, regardless of the drug's effect on the immune system, and yet had no serum blocking activity.

Sinkovics: In this particular situation, the same lymphocytes were tested against rhabdomyosarcoma cells treated with the 3 serum samples. The lymphocytes were collected earlier, were activated on rhabdomyosarcoma cells to which they responded violently, and then were retested.

Levy: I appreciate the technical difficulties, but still would emphasize that your results do not question the role of blocking factors in your patient's clinical course but merely show that depression of cell-mediated immunity, here with a drug rather than an antibody, is bad for the patient.

Sinkovics: In our paper we describe this patient in more detail and show that her cell-mediated immunity, as measured in vitro, was not abrogated, while her tumor advanced; during this time, no blocking factors could be demonstrated in her serum.[32] It is very difficult to collect enough lymphocytes because there is commonly leukopenia in these patients. As shown in our paper, the patient's lymphocytes were also cytotoxic to rhabdomyosarcoma cells; her serum contained no blocking factors but her tumors continued to grow.[32]

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[32] Sinkovics J, Cabiness J, Shullenberger C: Disappearance after chemotherapy of blocking serum factors as measured in vitro with lymphocytes cytotoxic to tumor cells. Cancer 1972. In press.

E. Klein: While the effect of serum for blocking lymphocyte-mediated cytotoxicity in vitro has been extensively studied and its significance for in vivo phenomena is greatly emphasized, less attention is paid to another mechanism by which antibody-coated cells can be killed by nonsensitized lymphoid cell population. This has, however, been demonstrated in several systems. When using target cells coated with different concentrations of antiserum, we found in the murine sarcoma virus (MSV) rat system that, depending on the antiserum concentration, lymphocyte cytotoxicity can be influenced in opposite directions. High concentrations could give an augmented cytotoxicity, whereas lower concentrations resulted in blocking. I would like to emphasize that, like in the H-2 system and in the adenovirus-induced tumor antigen system, sera sampled from nontumorous animals, such as MSV-immunized and regressor ones, could also have blocking effects. The assay was microcytotoxicity with 48 hours' incubation. The effector cells has low potency. In further studies concerning these 2 serum effects, Dr. R. Kiessling in our laboratory used a shorter assay: ^{51}Cr-labeled lymphoma cells were coated with antiserum (H-2 specificity) and exposed to normal or immune spleen cells for 4 hours. The immune spleen cells were usually more efficient in killing than antibodies and normal spleen cells. The former effect was somewhat inhibited when the target cells were coated with antibodies. There is good evidence that the cell compartment responsible for immune cytotoxicity is thymus derived, while the antibody complexed cells which are damaged by bone marrow-derived lymphocytes. Thus cytotoxicity registered by an immune population in the presence of antibodies could be expected to depend on the relative contribution of these mechanisms under the conditions used in the experiment.

McCoy: Dr. Klein, did you ever use any of those sera in passive transfer experiments?

E. Klein: No.

Levy: We have similar results in the human system and can block a cell-mediated reaction with anti-ABO isoantisera against the target cells. We find a very prominent concentration dependence in all our blocking studies. We generally run 3 dilutions of blocking sera, 1:2, 1:5, and 1:10 or higher. At 1:2 there is a blocking effect with both control and experimental sera. At 1:5 to 1:10 the so-called "specific" blocking activity of the experimental serum is evident but control serum activity is nil. Above 1:20 only rare sera had significant blocking activity.

Herberman: Are all of your experiments done by precoating the target cells and then washing before addition of lymphocytes?

Levy: Yes, we remove the blocking sera after 45–60 minutes at 37° C.

Herberman: How much do you wash your cells after you let them stay with the sera?

Levy: We wash the cells before addition of the sera but not at all after.

Herberman: What are your procedures, Dr. Klein? Do you wash your cells after incubation with serum?

E. Klein: The cells are washed once; however, washing in the microplate is not very efficient.

Mavligit: Dr. Levy, what would happen if you left the serum tested for blocking in the microtiter plate during the whole period of (40 hr) cocultivation with lymphocytes?

Santos: Do you always have complement in the system? If you do not, what does it mean, really, physiologically?

Levy: Dr. Mavligit, we are not pretending to recreate the in vivo state in a microtest plate! When we absorb blocking autoantisera with tumor cells, the absorption curve flattens out in 30–45 minutes; so whatever gets on the cell does so within the 45- to 60-minute incubation of our assay. As to keeping the antisera in the well during the entire test, Dr. B. Amos and his colleagues showed that, at least in their mouse tumor system, the antibody came off the cell during a 37° C incubation. If this was due to an antigen capping and endocytosis phenomenon, as reported by Drs. Raff, Kourilsky, and others, then prolonged incubation in the presence of immune serum might confuse the result by stimulating antigen endocytosis and producing spuriously high blocking activity. Of greater concern to me than the length of our incubation time is the dichotomy between the in vivo and in vitro blocking studies with regard to the required antibody dose. Do we mimic with our high concentrations of serum the effects produced by Dr. N. Kaliss and others with minute serum doses? We are trying to reproduce in vitro the low-dose blocking phenomenon by studying the effect of various immune serum concentrations on the development of primary antitumor cell-mediated immunity.

Herberman: Dr. Levy, to clarify a point you made earlier, can you specifically absorb out the blocking factors with the appropriate target cells?

Levy: Yes.

Herberman: Have you done the absorption with other kinds of tumor cells and shown that they do not remove blocking activity?

Levy: We can absorb out the anti-glioma blocking activity with autologous glioma cells, but not with autologous normal glia cells. We have not tried allogeneic tumor cells or fibroblasts.

Rubin: One thing is not very clear. It sounds as if you are saying that there are only one type of antibody and one type of antigen involved and, hence, the time you collect the sera is irrelevant. I think Dr. Klein's point is well taken, because if the blocking phenomenon is to mean anything, its detection and significance will depend on when you take the sera and what the clinical status of the patient is. An example of this, which may be relevant to the detection of the human blocking phenomena, is found in the MSV and the AKR tumor rat system. As known, if you inject virus or tumor cells to produce antisera in these rats, you find cytotoxic and other neutralizing types of antibodies in the sera. However, these sera do not contain antibodies that inhibit the polymerase. Furthermore, the only natural situation under which antibodies that inhibit polymerase activity are found is in tumor-bearing rats. This observation tells you something that has to be investigated in depth: What is going on between the tumor and the host? Is virus present? What is happening in terms of the kind of antibodies being made and will they block or not?

Oldham: Dr. Levy, do your blocking sera also block the "autoimmune" types of reactivity you described earlier?

Levy: The blocking sera do affect the "nonspecific" activity of control lymphocytes against the tumor cells. In 46 studies there was a 15–20% inhibition of tumor cells (relative to medium controls) by control lymphocytes. In 32 of these there was a diminution in the control effect in the presence of blocking sera. In 6 cases, however, the blocking sera actually augmented the control lymphocyte effect. I wonder if this latter observation was due to lymphocyte-dependent antibody in the blocking sera. We currently have a simple means of eliminating at least some, if not all, B-cells from peripheral blood and will repeat the experiments using the 6 sera with B-cell-depleted lymphocytes. It is very important to eliminate effects related to lymphocyte-dependent antibody because anything that augments normal lymphocyte killing will diminish the calculated relative percent inhibition by experimental lymphocytes and be falsely interpreted as blocking. We do not see an effect of blocking sera on normal target cells.

Oldham: I want to clarify one thing. Do you have the blocking sera that will enable you to remove the 10–15% of the nonspecific activity that occurs in 50% of your experiments? Am I clear on that?

Levy: Yes, the nonspecific reactivity of normal lymphocytes against tumor cells, but not the activity of patient lymphocytes against autologous glial cells.

Cohen: I have recently been studying the abrogation of cell-mediated cytotoxicity by hyperimmune serum using the ^{51}Cr cell-mediated assay. One disturbing finding has been the consistent blocking of cytotoxicity by normal serum when included in the reaction mixture in concentrations of $\geq 10\%$. Has anyone experienced this effect in any assay system and has he investigated it to any extent?

Levy: I have no direct answer to that question. When we prepare pseudoglobulin fractions of the patients' sera, we preserve the blocking activity. Similar fractions prepared from normal sera that had blocking activity at 1:2 do not block. I don't know whether this is an effect of elimination or simply dilution of the "nonspecific" blockers in whole normal serum.

Golub: Relevant to that, Dr. Brondz published data in the Russian literature showing blocking activity in normal sera in the mouse alloantigen system. The blocking factor was found in the α-globulin class and had a very strong blocking activity in undiluted serum. This may be contributing toward some of the things we have been discussing.

E. Klein: In a paper, human whole serum was shown to inhibit not only mixed lymphocyte reactions but also transformation by PHA or other antigens.

Levy: I think you are referring to Dr. Buckley's work. If so, these were not normal sera. Two were from multiparous women and two from immunized donors.

E. Klein: Yes, but it inhibited her own reactivity.

Levy: Although her blocking sera did not contain antibodies cytotoxic to autologous cells, she did not exclude the possibility of noncytotoxic autoantisera. Antibody against the *MLR* locus gene product could account for her results. In general, there seem to be both specific and nonspecific blocking antibodies. Dr. Buckley has evidence for both types and is carrying out absorption experiments to distinguish the two.

E. Klein: She has collected sera from the same subject and then assayed for HL-A antibody. She showed that the kinetics were not the same for blocking effect and cytotoxic antibodies.

Levy: Dr. Buckley compared the times of appearance of the blocking activity and a general lymphocytotoxic activity in 2 sera. In one serum the cytotoxic activity appeared before the blocking activity, but in the other both activities appeared simultaneously. In the latter serum, however, there was a temporal separation of some of the blocking effects and cytotoxic effects against specific individual's cells.

G. Klein: Would you not agree that a population of immune lymphoid cells will contain, among other things, *a*) specifically immune B-cells, *b*) normal B-cells that may kill antibody-coated target cells, and *c*) immune T-cells. The relative role of these compartments in target cell killing may vary in different systems. For example, Drs. P. Golstein, H. Wigzell, and others in our laboratory found recently that the killing efficiency of cell populations educated against a foreign H-2 complex remained unaffected when the B-cells were removed by passage through IgG-anti-IgG columns. Drs. E. Lamon and Wigzell now have preliminary evidence, however, suggesting that the situation may be different in tumor-associated antigen systems where B-cells may play a considerable role in target cell killing. I wonder whether work on the H-2 and HL-A systems may not create an optical illusion about the all-important role of T-cells, due to the fact that the strongest T-cell stimulator locus that bears the major responsibility for the unprimed MLC test is closely linked to H-2 or HL-A. Once we leave the ninth chromosome in the mouse, the stimulation may be quite different and T-cell killing may be only a modest part of the whole story.

Levy: Do you have any evidence from your fusion studies for linkage of any tumor-specific antigens to activity on the histocompatibility chromosomes?

G. Klein: No, we don't, but we did not look for this specifically.

Levy: You could be the one to do it with your fusion studies.

G. Klein: It would certainly be possible.

Levy: In the work on B-cell-mediated cytotoxicity, complement dependence has not been fully excluded. Mere exclusion of unheated serum from the system does not consider that some components remain intact after heat and others may reconstitute with time, nor that certain components are synthesized within the system itself. These experiments must be performed in the presence of specific complement inhibitors and with sera depleted of various complement components.

G. Klein: The main finding is that unseparated immune lymphocyte suspensions kill target cells without the addition of extraneous complement. If B-cells are removed, there is much less killing. B-cells may make complement components, of course, but this is quite unexplored.

Herberman: Dr. O'Toole, could you comment on your studies?

O'Toole: Removal of B-cells from human blood has virtually no effect on the PHA response, while antibody-induced cytotoxicity is markedly reduced.[33] Passage of lymphocytes from tumor-bearing patients through B-cell-retaining columns did reduce cytotoxicity in some patients' lymphocytes but not in others. Both types of columns gave reduced cytotoxicity in some experiments.

Herberman: When you say both columns, what do you mean?

O'Toole: The control column was coated with human IgG, but the specific B-cell-retaining column had additionally rabbit antihuman IgG.[34]

Herberman: Several groups have reported that mouse B-cells stick to glass beads. Do you think that is the explanation?

O'Toole: It's quite possible.

Herberman: Have you tried to use something like EAC rosettes or some other measure of B-cells in the eluates from the columns?

O'Toole: I understand that Dr. Wigzell has done this.

E. Klein: Dr. Wigzell usually tests for it. Using the glia cells as targets, we selected donors with cytotoxicity and Dr. Wigzell made cell separations. Comparing the control column (which contains only IgG) and the proper column (with the anti-human IgG), we could not see any effects on the cytotoxicity.

Levy: T-cells also have been shown to bear surface immunoglobulins, albeit in smaller amounts than B-cells. Possibly your columns are also removing some T-cell populations?

Herberman: Does anyone else have any particular comments to make about blocking, or B- versus T-cells?

Oldham: I'd like to ask a question unrelated to B- or T-cells. Is there any correlation between blocking

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activity in the cytotoxic assay and blocking activity in a stimulation assay as in the MLTC assay and have any cross-absorptions been done to show specificity? Does anyone have data on that?

Santos: I didn't get a full statement from Dr. Levy. Does anyone have sera that do not fix complement at all and are a blocking sera? The suspicion has been raised that one serum can do everything and you can manipulate it in your test systems. Nobody has taken a stand and if they have I haven't heard. If you don't have evidence that these sera are really clearly different or if you have other feelings I would like to hear it.

Levy: We do have sera from tumor patients that are not cytotoxic but have blocking activity. It is still possible, however, that these sera do fix complement in the presence of tumor cells but produce no detectable cytotoxicity. Such sera do block in the presence of carrageenin, however. We have also treated unheated blocking serum with cobra venom factor, then heated the serum at 56° C for 45 minutes, and found no alteration in blocking activity.

Santos: One can immunize an animal and get a very good cytotoxic response using a particular cell line from that animal, but then, using another cell from the same animal, one will not get any killing to speak of, at least by the usual tests. I think that it's a terribly important question to answer, whether or not there is a blocking antibody.

Herberman: Let's ask that question in a broader sense. Does anyone have information as to what blocking factor is by separation or by other characterization?

Levy: We have done several experiments that showed blocking activity in pseudoglobulin fractions and in diethylaminoethyl-cellulose eluates of blocking sera. Only one IgG column fraction had blocking activity. Thus at least one component of one form of blocking activity was probably IgG. It does not exclude the possibility that this IgG combined with soluble antigen after addition to the target cells and formed a blocking antigen-antibody complex. Nor does it exclude the possibility that antigen alone can have blocking activity apart from that detected in these studies.

Herberman: Does anybody else have evidence on what the blocking activity is?

Levy: A comment apropos Dr. Santos' remarks is related to experiments done several years ago showing exposure of antibody to pH 4 or lower irreversibly altered the Fc fragment while it left the combining sites intact. Thus any low pH elutions of "blocking antibody" may actually be elutions of cytotoxic antibody now unable to fix complement.

Rubin: Has anybody examined the 7S antibody, because what you say about needing it at a high concentration fits in with the optimal conditions for complement fixation by IgG? It is cytotoxic at high concentrations because you have more doublets formed on the cells and if complement is present, cell lysis occurs. As you dilute the 7S antibody—if that is the only type of antibody present—you can still add the same amount of complement and not lyse the cells. Under these conditions, you do not have complement-fixing, cytotoxic sites generated on the surface. But the antibody is still there. You can pick it up by adding anti-7S antibodies.

Santos: I didn't want to demean the data of the Hellströms or the preliminary data with correlations, but rather the interpretations. I'm afraid that, even in some of their writings, they have interpreted too much, and a lot of people have come away with the idea that this is the way that it is. I think that it is quite open. It correlated, sure, but one should be very careful before one says exactly why. I think the experiments of Dr. McKhann and your comments about the B-cells are very interesting, and I wonder what would happen if we put his attacker cells through a column and set that up for an in vitro test. Does he have B-cells? It's interesting that that material does not affect plasma cell tumors, so I think that these are extremely preliminary data.

Sigel: I'd like to address my question to Dr. Klein. That was a very interesting slide you showed, where the immune lymphocytes are being blocked by the same sera which enhanced the activity of nonimmune lymphocytes. There are also nonimmune lymphocytes present; why didn't you find some degree of improved activity by the nonimmune population?

E. Klein: In the first experiment done in the microplate assay with rat lymph-node cells, we had a synergistic effect, the improved activity. The immune population alone had rather weak activity. In the other system in which the synergistic effect was not apparent the immune population was rather active; it caused about 80% ^{51}Cr release in 4 hours. This system was entirely different. The target cells were mouse lymphoma cells; the effector cells were spleen cells. Maybe the effect was already maximal. Also we do not know the extent of the different mechanisms: possible blocking of the T-cell effect or B-cell cytotoxicity of the antibody-coated cells. We only see the end result. Certainly other factors influence the cytotoxic effects; e.g., the presence of antigen-antibody complex can block the B-cell killing.

Sigel: You seem to have a new way to help resolve the questions of binding sites on the lymphocytes. Your findings imply that the sites on normal and immune lymphocytes either react with different antibody molecules or trigger different cellular responses when confronted with the same antibody. It would be important to find out whether one is dealing with different classes of lymphocytes or with qualitative or quantitative differences in membrane sites.

Oldham: This gets back to the same question I asked before. Has anybody found any specificity based on absorption of the blocking activity? Until you do that, you cannot really determine what is going on.

Herberman: Dr. Levy said that he could.

Levy: There is still another type of absorption experiment to be done and it should best be done by Dr. H. O. Sjögren. He postulates that the blocking antigen-antibody complex can attach one end to the target cell and the other to the immune lymphocyte. He, therefore, should

be able to absorb out this blocking complex with immune lymphocytes.

Sinkovics: We did not do anything with absorption, but we did something else which indicated the specificity of the blocking factor. We had a rhabdomyosarcoma patient's lymphocytes which were activated on rhabdomyosarcoma cells and normal donor lymphocytes activated on rhabdomyosarcoma cells. We treated them and tested them for cytotoxicity, and the patient's lymphocytes were blocked by rhabdomyosarcoma-specific blocking factors. The normal lymphocytes which gained cytotoxicity upon activation against the rhabdomyosarcoma cells were not blocked. From this we concluded that the blocking factor coats the tumor antigen and prevents the access of those lymphocytes which are tumor-antigen committed. The normal individual developed a lymphocyte population which was histocompatibility-antigen committed and continued to have access to the target cells because the blocking factor specifically coating the tumor antigens does not coat the histocompatibility antigens.

Levy: Do you have evidence that the normal cells' activity was directed against histocompatibility antigens?

Sinkovics: The only thing is that the blocking factor did not block the normal individual.

Levy: Do these lymphocytes kill normal cells autologous to the tumor target cells?

Sinkovics: No, we did not have fibroblasts.

Levy: You may have seen the antibody-dependent B-cell-mediated activity alluded to earlier with intrinsic antibody production.

Sinkovics: What is your explanation for this phenomenon?

Levy: Perhaps blocking antibody has no effect on B-cell-mediated killing.

Sinkovics: But the B-lymphocyte killing is still theoretical at this time.

Levy: I think it's a real phenomenon, though its independence of the complement system has not been well proved.

PART II

MIGRATION INHIBITION ASSAYS

Studies on the Detection of Cellular Immunity to Human Tumors by Inhibition of Macrophage Migration [1]

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SUMMARY—The indirect migration-inhibitory factor assay in man correlates well with skin test results to soluble protein antigens. This assay, however, revealed no definitive evidence of cellular sensitivity to carcinoembryonic antigen in 9 patients with colon cancer. Inhibition of migration by autologous tumor antigens has been observed in 4 of 7 patients with breast cancer, but more patients must be studied before the significance of this finding is known.—Natl Cancer Inst Monogr 37: 135–137, 1973.

THIS SYMPOSIUM is devoted to reviewing in vitro techniques for detecting tumor immunity in man. Elsewhere in the Symposium, attempts to measure the proliferative response of tumor immune lymphocytes and the cytotoxic effect of sensitive lymphocytes have been reviewed. Our attempts to detect cellular immunity to human tumor rely on another aspect of lymphocyte function, the production of soluble lymphocyte mediators in response to lymphocyte stimulation by antigen. This assay has the advantage of measuring a soluble lymphocyte product, migration-inhibitory factor (MIF), which can be shown to be a good in vitro correlate of cellular immunity in experimental animals and in man. However, like the other available techniques, the application of this method to the detection of tumor immunity is still beset by many problems.

Inhibition of macrophage migration was originally observed in migration from a splenic explant but, as modified by subsequent workers, the technique consists of packing peritoneal exudate cells from sensitized animals into capillary tubes and observing the migration from the packed cell surface after the tube has been cut at the cell liquid interface (*1–3*). Adding antigen to the media results in inhibition of migration of macrophages, the extent of which can be quantified by comparison with migration of macrophages to which no antigen has been added.

Evidence that this in vitro assay is a good correlate of cellular immunity in vivo comes from several sources (*4*). In the guinea pig and in man, inhibition of macrophage migration correlates with the delayed skin reaction to the same antigen. Hapten-carrier specificity similar to that needed for in vivo cellular immunity is a requirement of this system (*5*). Except for scorbutic or newborn animals, positive migration assays without positive skin tests have not been demonstrated.

Cell-free supernatants from antigen- or mitogen-stimulated lymphocytes contain a number of lymphocyte mediators (*6, 7*). One of these mediators, MIF, can be detected by its effect on normal guinea pig peritoneal exudates. In man, the indirect migration assay depends on detection of this mediator in supernatants from antigen-stimulated lymphocytes by a convenient indicator system such as guinea-pig peritoneal exudate cells. This should be contrasted with the leukocyte migration assay in which periph-

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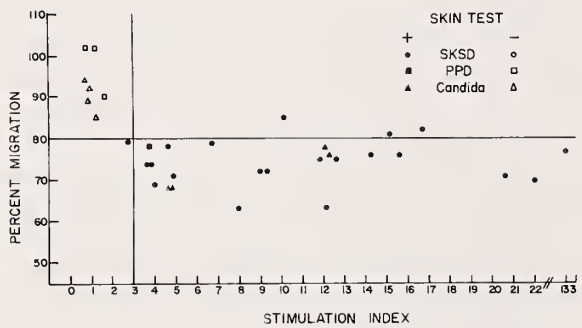
[2] Investigator, Howard Hughes Medical Institute.

[3] Supported by a Massachusetts Arthritis Foundation grant.

eral blood leukocytes are used containing both indicator cells and the sensitive lymphocytes. Presumably, MIF produced during the assay by lymphocytes stimulated by antigen inhibits migration of the indicator cells. Although each of these systems has some advantages, only the indirect assay will be discussed.

As developed initially by Thor et al. (*8*) and then by Rocklin et al. (*9*), the indirect migration assay has been shown to correlate with skin test results to the same antigen. As shown in text-figure 1, the agreement between the MIF assay, lymphocyte transformation, and skin test results was good. No false positive results were obtained. However, the incidence of false negative assays, in which the migration assay was negative despite a positive skin test to the same antigen, was from 10 to 20% of assays. To reduce the false negative results, the assay procedure has been modified in the following manner: Lymphocytes are collected over a Ficoll-Hypaque gradient (*10*) and incubated for 3 days with or without antigen at a final concentration of 5×10^6 cells/ml. After incubation, dialysis is completed in 2 rather than 3 days and the 5-times concentrated supernatants are then assayed on guinea pig macrophages as already described (*9*).



TEXT-FIGURE 1.—Correlation of lymphocyte transformation and inhibition of macrophage migration with the results of skin tests. Migration index: $\leq 80\%$ is positive. Stimulation index: ≥ 3.0 is positive. SKSD = streptokinase/streptodornase. PPD = purified protein derivative.

In our initial attempt to detect cellular sensitivity to tumor antigens, we chose to study the carcinoembryonic antigen (CEA) of colon cancer because a single, highly purified, CEA preparation, kindly supplied to us by Dr. P. Gold, could be used for all assays in the study (*11*). Thus, variable antigen toxicity was eliminated in this initial study. Blood lymphocytes were collected and assayed by the techniques described by Rocklin et al., with a final concentration of 10 μg/ml of CEA (*9*). The supernatants were collected daily for 3 days, with fresh media and antigen being replaced after each collection. In all, 9 patients with colon carcinoma and 10 normal volunteers were tested. The migration indices of these 2 groups were not significantly different (table 1).

TABLE 1.—*Inhibition of macrophage migration by CEA*

Patient with colon cancer	Percent migration
1	94
2	80
3	108
4	99
5	91
6	122
7	106
8	90
9	86
Normal volunteers	**Percent migration**
1	87
2	89
3	81
4	82
5	80
6	102
7	94
8	102
9	82
10	31

In view of the finding reported by Herberman and co-workers, that skin-reactive material in crude CEA could be separated from CEA by disc gel electrophoresis, these results are not surprising, especially since purified CEA was used in our experiments (*12*). There are, however, several reasons why these results do not exclude the presence of cellular sensitivity to CEA. First, the antigen may not have been used in sufficient concentration. Nonspecific antigen toxicity prevented the use of a concentration of antigen >10 μg/ml. Second, in view of the negative results, it is important to obtain a positive assay for some antigen to which cellular sensitivity is known to exist as an internal check on the technique. In these experiments, such internal controls were not consistently obtained.

More recently, we applied this technique to the problem of detecting cellular sensitivity to autochthonous tumors. Single tumor cells were prepared from tumor obtained at surgery by trypsinization or mechanical techniques. Tumor cells were mixed with autochthonous lymphocytes in ratios ranging from 1.4 to 13%, and the mixtures assayed for MIF by the modified procedure already described. To date, inhibition of migration has been observed in 4 of 7 patients with breast cancer in comparison to simultaneous controls, but more patients must be studied before the significance of this finding is clear.

In summary, the MIF assay in man correlates well with the results of skin tests to purified protein antigens. With relatively well-defined antigens, it has been possible to compare the lymphocyte function, as measured by production of MIF, of different groups of patients (*13*, *14*). With this assay, definitive evidence of cellular sensitivity to autochthonous human tumor antigens, however, has not yet been demonstrated.

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Problems With the Leukocyte Migration Inhibition Technique in the Study of Human Tumor Immunity [1]

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SUMMARY—The measurement of direct leukocyte migration is exquisitely sensitive to toxic factors present in antigen preparations from tumor tissue. In addition, a large amount of blood is required to perform relatively few assays. These disadvantages of the direct leukocyte migration assay severely limit the use of this technique in the study of human tumor immunity.—Natl Cancer Inst Monogr 37: 139–140, 1973.

IN 1970, Dr. John David and I reported on the use of a direct leukocyte migration-inhibition technique for the in vitro study of cellular immunity to soluble antigens (*1*, *2*). This technique represented only minor modifications of that reported previously by Sorborg and Bendixen (*3*, *4*). This method is based on measuring the inhibition of migration of a sensitized patient's peripheral leukocytes in the presence of antigen. The inhibition of migration was demonstrated to be antigen specific and to correlate well with skin-test reactivity to soluble antigens (*1–4*).

It seemed natural to attempt to apply this technique to the in vitro study of cellular immunity to human tumor antigens, and judging from the number of inquiries I have had regarding this technique, I gather many workers are attempting this. I thought it would be useful in a symposium such as this to discuss briefly my own experiences and views regarding this technique as applied to the study of human tumor immunity. My comments are limited strictly to the direct leukocyte migration procedure as Bendixen and his coworkers and I have reported it.

One limitation of this technique is its exquisite sensitivity to the toxicity of tissue-antigen preparations. The migrating cell population contains about 30–50% neutrophils, and the survival of these cells during the period of culture is marginal. These cells are susceptible to lysis in culture and can release large quantities of lysosomal enzymes which result in toxicity to other migrating cell types in the culture. It is thus important to distinguish the direct toxicity caused by an antigen preparation from its ability to cause immunologically specific inhibition of migration. This point has led to difficulties in the interpretation of some published results with this technique (*5*). I believe this problem of antigen toxicity is more severe in the presence of neutrophils. In our own studies of about 15 tumor patients, we used extracts from tumors prepared as described by Bendixen and his coworkers (*6*) and found nonspecific toxicity to be a problem in many preparations.

The direct leukocyte migration method also appears to require fairly high concentrations of antigen. In our studies on cellular immunity to purified protein derivative of tuberculin (PPD), in vitro concentrations of 0.1 mg/ml PPD were required to obtain consistent results. The need to achieve high concentrations of active tumor antigen and yet keep the concentrations of toxic substances at low levels presents a difficult prob-

[1] Presented at Conference and Workshop on Cellular Immune Reactions to Human Tumor-Associated Antigens, held at the National Cancer Institute, June 8 and 9, 1972.

lem. Significant purification of the tumor antigen appears to be a prerequisite for the use of the direct migration technique in the study of human tumor immunity.

Another problem of this procedure is the large amount of blood required for individual tests. With the capillary tube migration technique, about 15 capillaries could be filled with the peripheral cells from about 60 ml of whole blood. The test must be run at least in triplicate, several concentrations of an antigen preparation should be tested, and at least one specificity control is required in each experiment. Thus 15 assay tubes/60 ml of blood does not allow for much flexibility in the design of experiments. Extensive longitudinal or cross-specificity studies are also precluded by this large blood requirement.

Because of these problems, it appears that the direct technique for measuring the inhibition of leukocyte migration as it has been described by Sorborg and Bendixen (*3, 4*) and us (*1, 2,*) is not now in a state where it can effectively be used for extensive studies in human tumor immunity. I think the method does have a place in the study of cellular immunity to purified soluble antigens.

Recent modifications in the procedure may help overcome some of these problems. Use of purified peripheral cells or cultured human lymphocytes as the migrating cell, if these work effectively, may help overcome the exquisite sensitivity of the technique to toxic antigens. Micromodifications of the method, such as those reported by Clausen (*7*), may be important in reducing the amount of blood required for individual tests.

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PART II

MIGRATION INHIBITION ASSAYS

DISCUSSION

OUTLINE OF DISCUSSION—MIGRATION INHIBITION ASSAYS

I. Assay method
   A. Direct versus indirect
   B. ? Migration of granulocytes
   C. Capillary tubes versus Clausen technique
   D. Types of controls used
   E. Methods to determine if particular test is technically satisfactory
   F. Formula for calculation of results
   G. Is the purified protein derivative of tuberculin system the best model?
   H. Antigen mixed with migrating cells or in media

II. Preparation of migrating cells
   A. Amount of purification necessary or desirable
   B. Effects of heparin, Ficoll, etc., on migration

III. Antigens used
   A. Whole cells versus antigen extracts
   B. Types of extracts

IV. Serum blocking

M. Sigel: What antigen did you use, Dr. Santos?

G. Santos: We used the leukemic blasts, the whole cell. Since the frequency of the response in the family members is the same as the chromium release, you can't make any conclusions. We may be missing a lot. If you think our controls are reasonable, then these tests may mean something. I don't really know which is the best control to use, but we do use normal people. In our control of incubating the cells separately and then combining the supernatants, we have not had false positives.

W. J. Martin: You mentioned a correlation with some of your patients, that you have a chromium-release assay positive and also macrophage migration-inhibition factor (MIF) production. If you just lysed some of your leukemic cells, would that release a factor that would inhibit migration inhibition?

Santos: What he's pointing out is that, if the chromium release was positive, it should kill the cell and give a negative; I don't know if we really have the answer to that.

G. Mavligit: We have used KCl-extracted tumor antigen from colon carcinoma in the direct leukocyte migration test. The methodology was described in detail elsewhere.[1] The results are shown in table 1. The migration of patient's leukocytes was inhibited in the presence of the antigen while normal donor's leukocyte migration was not inhibited. This suggested that the inhibition of patient's leukocytes was specific and not a result of a toxic effect of the antigen on the migrating cells. The correlation in this case with lymphocyte blastogenic response and positive delayed cutaneous hypersensitivity reaction to this antigen suggests that we have probably detected cell-mediated tumor immunity. More studies need to be done before the leukocyte migration can be established as a reliable in vitro test for detection of cell-mediated tumor immunity. Until then, any interpretation must be done with caution.

TABLE 1.—*Leukocyte migration test—migration indices of patient's and normal donor's leukocytes with various antigen concentrations*

Antigen concentration	Patient's leukocytes	Normal leukocytes
Control	1.0	1.0
100 μg/ml	0.9	0.98
330 μg/ml	0.75	1.05
1000 μg/ml	0.45	1.42

R. Herberman: Just for clarification, Dr. Mavligit, the KCl extracts that you were using were just soluble extracts and not separated any further?

Mavligit: That is correct.

S. Rosenberg: This technique that you described is very different from the direct and indirect leukocyte techniques previously described. Have you demonstrated that this different technique can measure any immunologic response? Have you used this assay system to study the immunologic response to defined antigens, such as purified protein derivative of tuberculin (PPD) or Candida, in known sensitive and nonsensitive people?

Mavligit: We are doing that now. The purpose of this study was to demonstrate that solubilized tumor antigen may be a potential tool in the multifaceted immunologic evaluation of a cancer patient.

Rosenberg: That kind of information is essential be-

[1] MAVLIGIT G: Multifaceted evaluation of human tumor immunity using a salt-extracted colon carcinoma antigen. Proc Soc Exp Biol Med 140:1240–1245, 1972.

fore drawing any conclusions about your migration inhibition studies in a complex situation such as human cancer. You don't know whether your antigen preparations are active, nor whether the patients are sensitized, nor whether the technique can detect sensitization if it does exist. The first step should be to validate your technique in a well-defined system.

Mavligit: I agree with the principle.

Rosenberg: In human migration inhibition studies, several soluble antigen systems have been used to great advantage by several investigators. These include the tuberculin, Candida, and streptokinase/streptodornase antigens. Without these studies, what can you conclude about the 1 case you showed in which your migration studies correlated with the skin tests using your KCl antigen preparation?

Mavligit: You can conclude that those persons were probably presensitized.

Rosenberg: This may well be a simple modification of the migration inhibition technique, and we all know the technique needs modifications. But to begin studies with tumor antigens from tumor patients before the technique has been validated with a known system seems to be adding difficulty on difficulty, which will make the ultimate interpretation very difficult.

J. Gutterman: I believe Dr. Falk from Toronto has described in his direct migration system that the optimal antigen presented to the cells should be in a particulate form rather than a soluble form.[2]

Rosenberg: Actually it is a very different technique, in that the lymphocytes Dr. Falk prepares are purified by a different method; he does not run the migration in Pasteur pipettes but in Mackeness-type chambers. So there are really two major differences in the techniques, one of them, with preparation of the cells, being very critical.

W. Churchill: Our own attempts to use Ficoll-Hypaque in direct lymphocyte migration have not been very encouraging. In fact we have been unable to get any inhibition with experiments we have tried using soluble protein, antigens, and the lymphocyte fraction from a Ficoll-Hypaque gradient.

Santos: I understand why Dr. Rosenberg is critical, and he ought to be, particularly if he spent a year and then gave up the technique. I wonder if he thinks that it seems reasonable to look at a mouse that you know is immune biologically and, doing both direct and indirect tests, to directly compare the tests. Then looking at KCl extracts, you can compare it to a PPD response. Do you think that is a reasonable approach to determine if you have a good method or not?

Rosenberg: I would certainly think that animal tumor systems offer you the opportunity to do the kinds of controls that one could not do in humans. In fact, Dr. Churchill has done some beautiful studies using the migration inhibition technique in animal tumor systems. A problem, of course, is that techniques that work in the mouse may not be applicable to human studies.

Santos: Yes, all you have to do is look at the human skin test. Would you accept that? One could take the KCl extract and show not only by appearance but also by biopsy that the delayed hypersensitivity interpreted by the clinician was actually delayed hypersensitivity. If the correlation was good, would you accept it then? Let's say he studies 50 patients and he gets 10 of them, on whom he performs a biopsy, whose skin test is positive and the correlation with the MIF test is good.

Rosenberg: I would say that establishing a correlation between the migration inhibition technique and other measurements adds validity to the migration studies. Those other measurements need not be skin tests necessarily, but certainly this would be one good choice. I would, however, like to raise a point. This kind of preliminary study can be done so much more easily with known soluble antigens than with tumor antigens. Once the technique has been validated with simpler antigens, applications to tumor antigens could logically follow.

Santos: If a person shows a positive skin test to his tumor, one is not worried as to whether that means it is tumor specific but, in fact, that they react to it.

Rosenberg: But it may be only inflammatory on a nonspecific basis.

Santos: Right, but there are some very good immunopathologists and very critical ones. You certainly could look at that very critically with a biopsy. Would you agree to skin reactions or not?

Rosenberg: Immunologic specificity must be demonstrated regardless of the histologic pattern. If normal, nonsensitized humans gave "positive" skin tests, then the interpretation of the studies would be markedly altered.

Mavligit: Unfortunately one cannot take a tumor antigen and inject it into a normal donor. The risk of transmission of malignancy by a virus, or a viral product, is still ethically unacceptable.

Rosenberg: That is exactly my point, and that is why I am emphasizing the need for preliminary studies with your modified technique on simpler antigen systems.

Herberman: We'll get back into this with the skin tests. I think that some control skin tests can be performed.

Martin: I would like to caution against overinterpretation of a positive assay. Regardless of the nature of the assay, providing one has adequate specificity controls, a positive assay indicates only that tumor immunization has occurred. To extrapolate the implication of a positive assay in experimental animal systems to man would seem premature, especially when individual assays might be mediated by several quite distinct immune processes which may be variously correlated with the presence of other immune modalities.

I would also like to comment, Dr. Churchill, that I am surprised with the rather weak correlation between skin reactivity and the degree of MIF production. Their common presence may be merely coincidental rather than the reflection of a common underlying mechanism. The

[2] ZABRISKIE J, FALK R: In vitro reactivity of lymphocytes to particulate and soluble antigens. Nature (Lond) 226:943–945, 1970.

presence of false negatives also argues for the existence of independent processes.

Herberman: The point that Dr. Martin is making is very important. There are 2 things that we have to keep separate: 1) whether the test that one is doing has any immunologic specificity and 2) whether the test is a measure of cell-mediated immunity that operates by the same mechanism that delayed-hypersensitivity skin tests do. I think that Dr. Rosenberg may also have the second problem, despite the kind of data that he has with the PPD system. One could still raise the question as to whether his migration assay is a measure of delayed hypersensitivity, even though he has a fairly good correlation with skin tests. He might be measuring antibody-mediated effects rather than a complete correlate of the skin tests, in that patients who are skin test-positive to PPD could also have antibodies and other immune phenomenon going on at the same time.

Churchill: In answer to Dr. Martin, the system as has been set up in Dr. David's laboratory has essentially no false positives. The correlation between positive assays and positive skin tests is there. It is true that we do have some false negatives, and our feeling is that it's probably a problem in the indicator system we are using. Guinea pig macrophages are not very efficient at detecting human MIF

Martin: The existence of negative skin tests with positive MIF production in tumor-bearing hosts could, conceivably, be due to the existence of blocking factors suppressing in vivo reactions.

Churchill: I am not aware that anyone has shown that blocking in vitro has resulted in negative skin tests in vivo.

Martin: The point was raised merely to indicate the need for better understanding of the mechanisms underlying the various in vivo and in vitro assays. For example, a definition of the in vivo role of MIF and the development of more quantitative and specific assays of its in vitro production would be useful.

Churchill: Everyone in the field in a sense agrees with you. We all believe that the available assays have real limitations and we would like to develop better ones.

J. Holland: Does the supernatant containing the lymphotoxin produce a skin change itself on intradermal injection as compared with the controls?

Churchill: Partially purified supernatants from stimulated guinea-pig lymphocytes contain a factor which causes a skin reddening reaction, the histology of which resembles the morphologic features of a delayed-hypersensitivity skin reaction.[3] But this type of experiment has not been done in man.

Holland: Why would you hesitate to extrapolate that to man? It would make a marvelous control for delayed hypersensitivity.

Churchill: As far as I know, that has not been done.

Santos: I agree that it's nice to have someone working on more defined systems. It depends on whom you are talking to as to whether you have a defined system or not. If you have done your correlations with your skin tests, the correlation of what is happening inside that patient is a little tough. For years we've accepted biopsy-proved skin reactions with the right time course for delayed hypersensitivity. If you want to throw it out, then you're redefining it. It's fine to raise the question, and we need to do that every so often but you're being just a little too severe by saying that you must have a more defined antigen. If you talk to a physicist and you need anything more than the atom, it's so complex that he won't be able to understand it. I'm just trying to make a point.

Gutterman: I would like to show human MIF production to soluble leukemia antigen prepared by 3M KCl extraction. The assay used is as that described earlier by Dr. Mavligit. I will say at the outset that we are not certain that this assay measures MIF. This particular assay is quite similar to that published recently by Dr. Wolberg.[4,5] He did separate his cells differently from the method we are using (Ficoll-Hypaque). Dr. Wolberg thought that maybe his assay was measuring a toxic effect on leukocyte migration.

Five patients with acute leukemia and 1 patient with blast crisis of chronic myelogenous leukemia have been studied. A migration index of ≤ 0.8 was considered to be significant inhibition compared to the control. Three of the four acute myelogenous leukemia (AML) patients had a positive MIF sometime during their remission. The patient with acute lymphocytic leukemia, the patient with blast crisis, and 1 patient with AML had no significant inhibition with autologous leukemia antigen. Only 1 of the 3 positive MIF reactions had a positive skin test. The disturbing feature of these very preliminary data is that several normal controls have also had inhibition of their leukocytes when incubated with one leukemia antigen. We may indeed be seeing a nonspecific toxic effect by the leukemia antigen on migrating leukocytes.

Because of the difficulty we have had with the direct assay, we have started experiments with the indirect method using guinea pig macrophages as described by Rocklin and coworkers. Although this assay certainly is more tedious, it is well accepted and, hopefully, will prove to give specific immunologic information.

Herberman: At what protein concentrations were your tests done?

Gutterman: These were from 10 to 200 μg/ml.

Herberman: Have you gone through this same pro-

[3] Bennett B, Bloom BR: Reaction in vivo and in vitro produced by a soluble substance associated with delayed-type hypersensitivity. Proc Natl Acad Sci USA 59:756–762, 1968.

[4] Wolberg W, Goelzen M: In vitro assay of cell mediated immunity in human cancer: Definition of leukocyte migration inhibitory factor. Nature (Lond) 229:632–634, 1971.

[5] Wolberg WH: Inhibition of migration of human autogenous and allogeneic leukocytes by extracts of patients' cancers. Cancer Res 31:798–802, 1971.

cedure with normal leukocytes from the same patients or from other patients?

Gutterman: No, but we are now making KCl extract from normal leukocytes for our assays.

Herberman: So at the moment, if we get back to the point that Dr. Rosenberg was raising, how strong is the evidence that what you are seeing is immunologic? Also what is the evidence that the monocytes are being inhibited?

Gutterman: I say that there are none. I only presented this to show what our experience to date has been. I will be showing data that suggest our antigen is specific because the normal donors do not respond, and that the patients do respond by in vitro blastogenesis. But with this assay for MIF, we can make no conclusions yet. The indirect method of Rocklin will possibly be required.

E. Klein: In a number of tumor cases the group of Lacour in Paris reported (*International Journal of Cancer*) inhibition of leukocyte migration by autochthonous tumor extracts. Healthy subjects did not react. Only a few cross-reactivities were seen in melanoma cases.

Churchill: In a recent paper[6] positive inhibition of migration assays was observed when saline extract of lymphosarcoma was added to leukocytes from patients with lymphosarcoma.

Herberman: With all the published reports and from the comments made here, it sounds as if most of the positive results with tumor systems have been with direct migration assays. Although the indirect system seems to work fairly well with PPD and other soluble antigens, it has not been very successful with tumor antigens, except for Dr. Santos' study.

Churchill: I think that's basically correct. The data we have are not enough to convince me that we have definitely measured a positive result and I don't know of any published data which convinces me.

Herberman: Would someone like to comment on the type of assay that Dr. Clausen et al. have been doing with migration in soft agar? At least from what they've published, it seems more sensitive than the capillary tube migration and would potentially seem to be more direct and more straightforward. They claim that it could use smaller amounts of cells and antigens. This kind of assay, if it can be reproduced, might solve some of the problems that Dr. Rosenberg was raising. Dr. Clausen claims that he can get his migration effect with granulocytes separated from a Ficoll-Hypaque gradient. Dr. Rosenberg has some thoughts about what kinds of cells are migrating in his direct system. Would you like to comment on that?

Rosenberg: I have had no direct experience with Dr. Clausen's system. In the direct migration system that I have studied using total-blood leukocytes in migrating chambers, one does not see a migration inhibition of leukocytes even with a positive test using defined antigens.[7] That is, if one samples the outer halo of cells whose migration is not inhibited even in positive tests, these cells are all neutrophils; if one looks at the inner mass of migrating cells which are inhibited, they are primarily mononuclear cells. Thus, the direct test as performed in the capillary tubes does not inhibit migration of leukocytes but only mononuclear cells.

The method of preparation of the cells ends up with some increase in the percentage of mononuclear cells compared to the original amount of neutrophils.

Herberman: Is the PPD system the best model for development of a migration assay? This is particularly of concern since the recent report about the deterioration of PPD after a very short period of time. If you have problems with PPD, does this mean that the system is not going to work or be a valid test with other antigens, particularly tumor antigens?

Rosenberg: For our technique to work, we had to mix up our PPD fresh each day that we ran the test. If we prepared PPD and let it sit around for several days, we could not get consistent inhibition of migration. I don't think there is anything magical about PPD, although perhaps humans have less of a humoral response to that particular antigen than they have to other antigens and it might be a cleaner example of a hypersensitivity-type reaction. But I think there would be a variety of defined antigens that can be used. Another approach is to take an antigen to which humans have never been exposed, e.g., keyhole limpet hemocyanin, and do migration studies before and after immunization. This might be one way to try and validate the technique as an immunologically significant one. There are many different antigens or approaches that can be used.

Herberman: Most of the people doing the assays put the antigens into the incubation media rather than doing a preincubation of the migrating cell population with the antigen, and then putting the mixture into the tubes. Dr. A. Cochran, in Scotland, claims that he gets considerably better results by preincubating the cells with antigen.

Rosenberg: I've seen those data; it's a 1-hour incubation at 37° C. Then he takes his mixture of media, antigen, and leukocytes and he just uses buffy-coat leukocytes, spins them down into the capillary, and lets them migrate out into fresh media. He had fairly consistent inhibitions of migrations in the direct test with melanoma extract. It was not completely established that this material would inhibit melanoma cells but it seemed fairly good. Although quite preliminary, it looked very promising.

Herberman: Has anyone tried this type of preincubation? Theoretically this would make it a more sensitive system, having the cells come in contact with the more concentrated form of the antigen. If the antigen were only outside in the medium, the cells would have to

[6] Braun M, Sen L, Bachman A, et al: Cell migration inhibition in human lymphomas using lymph node and cell line antigens. Blood 39:368–376, 1972.

[7] Rosenberg S, David J: Inhibition of leukocyte migration: An evaluation of this in vitro assay of delayed hypersensitivity in man to a soluble antigen. J Immunol 105:1147–1152, 1970.

come out of the capillary tubes into the medium to meet the antigen.

Churchill: In the animal work I have done, I placed the tumor antigens mixed right in with the cells. With the Bendixen direct assay, we mixed the antigen in with the cells so that everything would have inhibited the controls as well to the same extent that the patient was.

M. Sigel: We did a few experiments in which we incorporated antigen with peripheral blood cells and placed the capillaries on agar to which antigen had also been added. With animal leukocytes this appeared to give better results than those of limiting the antigen to the agar.

Herberman: Is there information available on toxic or deleterious effects of heparin or Ficoll on migrating cells?

Mavligit: Two years ago, Kaltreider et al.[8] raised a serious question whether migration is not merely a gravity effect. We had an opportunity to study 2 patients 2 or 3 days after the administration of chemotherapy. To our surprise, there was no movement whatsoever of leukocytes out of the capillaries, suggesting indirectly that leukocyte migration is at least in part a functional process rather than totally a mere effect of gravity.

Holland: It would be easier to put a wedge under one edge of your dish to make it go uphill; it wouldn't be gravity at that time.

Mavligit: We have tried that and there isn't so much migration compared to the horizontal position.

Holland: Then there is a gravity effect?

Mavligit: When you have the culture media on top, fluid weight may press the migrating cells down, so that the vector of migration is now opposed by 2 vectors, i.e., the gravity of the fluid and the gravity of the cells.

Rosenberg: I have the general impression that migration always *starts* by cells falling from the capillary tube. If one looks after just a few hours, there is a massive emptying of cells from the tip of the capillary tube. Then the cells that have fallen out onto the coverslip have the opportunity to move or not to move. So very likely the actual migration is a combination of gravity and active migration. Clearly there are tests that could distinguish between those 2 factors.

Santos: Have you seen a stimulation of migration by antigen?

Rosenberg: In the 25 patients we studied with known positive or negative PPD tests, 1 patient with a positive skin test did not show migration inhibition. This patient had the strongest skin test of the 25 (measuring about 2½ cm in diameter after 48 hr). She showed a marked enhancement of migration which might itself have been due to some immunologic mechanism.

Santos: Do you find that the shape of the curves vary for people?

Rosenberg: If we go to >100 μg/ml of PPD, we begin to get toxicity in everybody.

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[8] Kaltreider HB, Soghor D, Taylor JB, et al: Capillary tube migration for detection of human delayed hypersensitivity: Difficulties encountered with "buffy coat" cells and tuberculin antigen. J Immunol 103:179–184, 1969.

PART III

LYMPHOCYTE STIMULATION

Storage Method for Leukemia Blast Cells [1]

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SUMMARY—A number of in vitro and in vivo assays have been used to demonstrate host immune reactivity to autologous tumor antigens. Autologous tumor tissue in some form is necessary to perform these assays, sometimes as viable whole cells. Because tumor tissue is not always available directly from the patient, a method of storage is required for continued testing. We have solved this problem in the acute leukemias with a method of freeze-storage that will preserve the blast cells in a viable form suitable for several assay systems.—Natl Cancer Inst Monogr 37: 149–151, 1973.

THE ABILITY of human lymphocytes to undergo blastogenic transformation upon exposure in vitro to autologous leukemic blast cells has been well documented (*1–3*). Other assays of cell-mediated immunity have also demonstrated host immune reactivity to neoplastic tissue (*1, 4*). These tests suggest that leukemic and other tumor cells have antigenic determinants that are peculiar and separate from normal tissue antigens. Some of these assays require viable, intact tumor cells as the source of tumor antigen. Because freshly explanted tumor tissue is not always available (e.g., when the patient is in remission), another source of tumor cells is needed for multiple testing. This problem has been attacked by *a*) the establishment of tumor cells in long-term tissue culture and *b*) freeze-storage. Continuous cell cultures have several inherent deficiencies. Not all cell types can be maintained for a long time, and those that can may be accidentally destroyed or become infected with either bacteria or viruses. There is also the continual possibility of random genetic change which would make test results difficult to interpret. For these and other reasons we elected to store large numbers of tumor cells, taken from individual patients, in liquid nitrogen. We have used these cells as a source of antigen for immune evaluation in several assays. Our techniques and results with studies of acute leukemia are presented here.

CELL SOURCE

When a patient is first admitted or admitted in relapse, leukemia blast cells are obtained for storage by 1 of 2 methods. The method of choice is leukopheresis. This, of course, requires the presence of blast cells in the peripheral blood. If there are no circulating blasts, the alternate method of collection is multiple bone marrow aspirations. Leukopheresis is preferred for several reasons. The numbers of cells that can be obtained are much greater, although somewhat dependent on the number of circulating blasts and the size of the patient. When there are few leukemic cells in the peripheral blood, the concentration can usually be increased during leukopheresis by adjusting the force and duration of whole-blood centrifugation. A force of approximately 200 × *g* is generally satisfactory to enrich leukocyte-rich plasma with blast cells (*5*). This also removes neutrophils which would later in-

[1] Presented at Conference and Workshop on Cellular Immune Reactions to Human Tumor-Associated Antigens, held at the National Cancer Institute, June 8 and 9, 1972.

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[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

terfere with freezing and thawing (*6*). When bone marrow aspirations are required, a standard technique is followed and the posterior iliac crests are used (*7*). This method presents problems in that the procedure is very painful to the patient, the number of blast cells obtained is usually low, and clotting of the specimen is difficult to control.

SPECIMEN PROCESSING

The cells are collected in heparin—5 U/ml for leukopheresis and 10 U/ml for bone marrow aspirates. Acid citrate dextrose may also be used (*8*). Contaminating red blood cells are removed by sedimentation with dextran (6% solution) at a concentration of 10% by volume. The leukocyte-rich plasma is removed and suspended in Eagle's minimal essential medium (MEM). Autologous plasma is used in the suspension. The concentration is always maintained at at least 10%. The final concentration of cells in the suspension should not exceed 1×10^7/ml.

FREEZING TECHNIQUE

The phenomena which injure cells during the course of freezing are complex and have been reviewed elsewhere (*9*). The probability of injury by ice-crystal formation and solute concentration can be reduced by a cryoprotective agent and a controlled rate freezing. Dimethyl sulfoxide (DMSO) is preferred as the cryoprotective substance and is used in a concentration of 10% by volume. The DMSO is added before the cell suspension is frozen in a diluted form. Concentrated DMSO should never be added to cell suspensions because the heat of solution can be great and potentially damaging osmotic stress would be created. Once the DMSO has been added to the cell suspension, it is rapidly divided into small Teflon bags and frozen (Cryogenic Container, code 2R2902, Travenol Laboratory, Inc., Morton Grove, Ill.). After DMSO is added to the cells, controlled freezing should start as rapidly as possible. DMSO is cytotoxic at above-freezing temperatures.

Leukocytes are best preserved by cooling slowly at 1–2° C/minute (*10*). This can be done with commercially available equipment (BF-4, Linde freeze controller, Linde Division, Union Carbide Corporation, New York, N.Y., and BV-4, Cryoson Technisch Laboratory, Middenweg 112, Middenbeemster-Holland). During the freezing phase, particular attention should be given to the heat-of-fusion period. For nucleated cells, the time spent in this phase should not be prolonged (*11*). When a temperature of −80° C has been reached, the cells should be transferred to a long-term storage facility (LR-1000, Linde liquid nitrogen refrigerator) which will keep the cells at −130° C to −175° C (vapor temperature of liquid nitrogen).

THAWING

Thawing should be rapid. We have found it efficient to agitate the Teflon container in a water bath at 37° C until the ice mass melts. Once thawed, the cells should be washed free of DMSO by slowly adding Eagle's MEM (with 10% plasma) in equal volumes for the first washing. The cells are similarly washed once again before final suspension for counting.

In general, this freezing and thawing process will reduce the total number of blast cells by 20–30%. More than 90% of the remaining cells are viable, as determined by trypan blue.

SELECTED USES OF FROZEN BLAST CELLS

Blast cells stored in this way have been used in the following tests: 1) Mixed leukocyte culture: It has been possible to use these cells to demonstrate lymphocyte blastogenesis as a measure of immunity to leukemia (*1*). The mitogenic effect has been shown to be specific rather than the result of a nonspecific mitogen produced or released during the cell processing (*12*). 2) Lymphocyte cytotoxicity assay: With ^{51}chromium as the label, patients and some relatives of patients with acute leukemia have been shown to possess cytotoxic lymphocytes that will lyse blast cells (*1, 13*). 3) Detection of a leukemia-associated antibody: Heterologous antibody produced in rabbits has demonstrated a leukemia-associated antigen that is present on fresh and frozen leukemic cells (*14*). 4) Steroid-binding protein: Cytoplasmic glucocorticoid-binding

proteins have been identified and quantified in leukemic blast cells (*15*).

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Cell-Mediated and Humoral Immune Response to Acute Leukemia Cells and Soluble Leukemia Antigen — Relationship to Immunocompetence and Prognosis [1, 2]

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SUMMARY—Concurrent studies of lymphocyte blastogenic response to autologous leukemia cells, of general immunocompetence, and direct membrane immunofluorescence with anti-immunoglobulin serum were carried out in 35 adults with acute leukemia. In addition, lymphocyte blastogenic response of autologous and allogeneic lymphocytes to soluble leukemia antigen was studied. Of 35 patients, 25 (72%) had positive blastogenic response to their own leukemia cells. Patients with acute myelogenous leukemia (AML) tended to have a greater blastogenic response to their leukemia cells than patients with acute lymphoblastic leukemia (ALL). Eight of nineteen patients with AML and one of five with ALL had complete or partial abrogation of this positive blastogenesis when the lymphocytes were cultured in autologous serum rather than allogeneic serum. Direct membrane immunofluorescence with anti-immunoglobulin serum showed bound IgG on the cells of 7 of 8 AML patients with the serum-inhibitory effect and 1 AML patient without the serum-inhibitory effect. There was facilitation of blastogenic response in autologous serum compared to allogeneic serum in 5 instances. A good prognosis was correlated with a vigorous blastogenic response, its inhibition or facilitation by autologous serum, and IgG bound to the cell surface. Of 7 patients, 6 had positive blastogenic response to autologous soluble antigen. Of these 6 patients, 5 also responded to autologous leukemia cells. Although allogeneic normal donor lymphocytes responded vigorously to leukemia cells in 6 of 7 instances, only 1 of 7 allogeneic lymphocytes responded to soluble antigen. Thus, presensitization apparently is required for in vitro blastogenic response to soluble leukemia antigen.— Natl Cancer Inst Monogr 37: 153–165, 1973.

DURING THE PAST several years, evidence has been increasing that tumor-associated antigens are present in various human neoplasms (*1*). In studying the immunologic response to human leukemia, Yoshida and Imai found that the sera of 71% of patients with acute leukemia gave a position immune adherence reaction with autologous leukemia cells (*2*). In contrast, using similar methods, Doré and co-workers found that only 22% of 140 patients with acute leukemia had evidence of antibody directed against

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autologous leukemia cells (*3*). Observing cell-mediated immunity, Oren and Herberman reported that most patients with acute leukemia had positive delayed-hypersensitivity skin tests to membrane preparations of their own leukemia cells (*4*). Cellular reactivity to acute leukemia cells has also been demonstrated in vitro by the mixed leukocyte culture (MLC) technique and lymphocyte cytotoxicity (*5–9*).

Of possible relevance to the apparent failure of this tumor immunity to control clinical cancer was the demonstration that serum factors in solid tumor patients may interfere with the in vitro cell-mediated immunity (*10*). The Hellströms have described a factor (presumably antitumor antibody) in the serum of patients with solid tumors which may block lymphocyte-mediated cytotoxicity to tumor cells (*11*).

Few studies have been designed to determine the role of serum factors in tumor immunity in patients with acute leukemia. Therefore, the current study was designed to determine the presence and possible interrelationship of humoral and cell-associated tumor immunity in acute leukemia. In addition, the interrelationship between general immunocompetence and specific tumor responsiveness was studied. Evidence for both humoral and cell-mediated tumor immunity was found (*12*). The presence of a vigorous lymphocyte blastogenic response to leukemia cells, its inhibition or facilitation by autologous serum, and the presence of immunoglobulin bound to leukemia cells were found to correlate with a good prognosis.

The technique for salt extraction of soluble HL-A antigens (*13*) has recently been applied to the extraction of tumor-associated antigens in animal tumors (*14*). We have applied similar techniques to the extraction of soluble antigens from human leukemia cells. These soluble preparations have been assayed in lymphocyte cultures with both autologous leukemic patients' lymphocytes and allogeneic normal donors' lymphocytes. These preliminary studies are reported here. In addition to demonstrating the feasibility of salt extraction of antigens from human leukemic cells, the current data strongly suggest that the lymphocytes' responsiveness in vitro represents specific tumor immunity.

MATERIALS AND METHODS

Thirty-five adult patients with acute leukemia had studies for membrane immunofluorescence with anti-immunoglobulin antisera blastogenic response and skin test reactivity to autologous leukemia cells. In addition, 6 of these patients and 1 patient with "blast crisis" of chronic myelogenous leukemia (CML) were studied for lymphocyte blastogenesis and skin test reactivity to soluble antigen.

Leukemic blast cells were collected from the peripheral blood of untreated patients on admission to the hospital. They were collected with the IBM or the Aminco blood cell separators as previously used to obtain lymphocytes (*15*). The red blood cells were removed by exposure of the collected cells (in their original acid-citrate-dextrose suspending medium) to 5 volumes of Tris-buffered ammonium chloride (*16*). The leukemia cells were centrifuged, washed, and then resuspended in RPMI 1640 with 20% fetal calf serum. This suspension was mixed with a 10% volume of dimethyl sulfoxide and frozen at 1° C per minute to −120° C in sterile glass ampuls at a leukocyte concentration of 5×10^7/ml and in a volume of 1 ml. The ampuls were stored at −310° C in liquid nitrogen. On the day of study, the ampuls were thawed rapidly at 37° C, washed 3 times in Spinner modified Eagle's minimal essential medium (MEM) (Hyland Laboratories, Los Angeles, Calif.), and resuspended in MEM at room temperature. The cells were counted in a hemocytometer chamber, and viability was determined by trypan-blue dye exclusion (*17*).

Extraction of tumor antigen.—The leukemia cells were collected as described above, and the red blood cells were lysed by Tris-buffered ammonium chloride for 15 minutes. Depending on the availability of cells, 3×10^8–3×10^{10} leukemia cells were used for antigen extraction. The method described by Meltzer et al. (*14*) was used with slight modifications. A total of 10 ml of 3M KCl in potassium phosphate-buffered saline (PBS) at pH 7.2 was added to every 3×10^8 cells in 25×200-mm screw-cap glass tubes. This mixture was equilibrated at 4° C for 16–24 hours, and the contents were then centrifuged at $100{,}000\times g$ for 60 minutes at 4° C.

This supernatant was dialyzed against 20 volumes of potassium PBS for 24 hours; the solution was changed every 8 hours. The dialyzed extract was centrifuged at 40,000×g for 15 minutes at 4° C. The supernatant was then concentrated by ultrafiltration at 4° C, and the concentrate was mixed with an equal volume of 3.8M ammonium sulfate for 1 hour at 4° C to precipitate the protein. This mixture was centrifuged at 40,000×*g* for 15 minutes at 4° C. The precipitate was redissolved in 5 ml of PBS, pH 7.2, and dialyzed against this buffer for 4 hours. The protein concentration was estimated by optical measurement at a wavelength of 280 nm (OD 280). The final preparation was sterilized by being passed through a 0.45μ Millipore filter (Millipore Corp., Bedford, Mass.); it was stored at 4° C.

For the lymphocyte culture and other lymphocyte studies, peripheral blood lymphocytes were collected, washed, and cultured as previously described (*18*). Cultures contained 1×10^6 washed lymphocytes, 1 ml of serum (either autologous or allogeneic), 2 ml of MEM, and various stimulants including leukemia cells, phytohemagglutinin (PHA-M-Difco Laboratories, Detroit, Mich.), streptolysin O (SLO-Difco), and allogeneic normal leukocytes. The stimulator leukemia cells were added in doses varying from 10^4 to 10^6 in half-log increments. Both unirradiated and irradiated cells were studied (4,000 or 12,000 R). Extracted tumor antigen was added in a dose range of 10–1000 μg/ml of culture. Appropriate controls were set up, consisting of unstimulated lymphocytes, unirradiated leukemia cells cultured alone, and irradiated leukemia cells cultured with irradiated lymphocytes. Cultures were incubated for 3–5 days at 37° C in an atmosphere at 5% CO_2 in air (PHA, SLO), 5 days for tumor antigen, or 7 days for MLC and tumor cells. Harvesting was done by addition of 2 μCi of ^{3}H-thymidine with a specific activity 1.9 Ci/mM for 3 hours (*18*) and by measurement of the acid-insoluble radioactivity by liquid scintillation counting (Schwarz BioResearch, Orangeburg, N.Y.).

Lymphocyte blastogenesis was measured as the net counts per minute (cpm) of tritiated thymidine incorporation per 1×10^6 cultured lymphocytes with the incorporation of the appropriate controls subtracted from that of the stimulated cultures. A 100% increase or 50% decrease in thymidine incorporation compared to the appropriate control was significant stimulation or inhibition at the 95% level of confidence (*19*), and a 300% increase or 75% decrease was significant at the 95% level of confidence. The stimulation index (SI) was defined as the cpm of a stimulated culture divided by the cpm of the appropriate unstimulated lymphocyte culture.

Membrane immunofluorescence was performed by the method of Möller (*21*) with specific animal antihuman IgG, IgA, or IgM purified by absorption to and elution from insoluble immunoabsorbent antigens (*20*). Leukemia cells were thawed rapidly, washed 3 times with MEM, and then incubated at room temperature for 60 minutes with either patient's serum, serum from a normal donor with the blood type AB rh-positive, or McCoy's medium (modified) with 30% fetal calf serum. These incubated cells were washed 3 times in PBS-McCoy's before being exposed to the fluorescein-conjugated antisera specific for human IgG, IgA, or IgM heavy chains as previously described (*22*). Leukemia cells were resuspended in the appropriate fluorescein-conjugated antiserum and incubated for 30 minutes at 22° C. After being washed 3 times in PBS-McCoy's, the cells were mounted in a solution of PBS and glycerin on glass slides and read immediately, or they were fixed with glutaraldehyde and read within 7 days. A reaction was positive when $\geq$20% of the cells showed surface membrane fluorescence with an antibody reagent against a single immunoglobulin class (100-cell count).

Skin testing.—Live autologous leukemia cells (0.1 ml) in doses from 10^4 to 10^6 in half-log intervals were injected intradermally. Delayed hypersensitivity was evaluated with soluble tumor antigen by intradermal injection of 0.1 ml of extracted antigen. No more than 0.33 mg of protein/0.1 ml was injected. The ability of patients to react to skin tests was also evaluated by a battery of established skin test antigens including dermatophytin, dermatophytin O, and Candida antigen or Monila mixture

(all from Hollister-Stier Laboratories, Downers Grove, Ill.) and given in 0.1 ml of a 1/20 dilution of the stock, streptokinase-streptodornase (Varidase, Lederle Laboratories, Pearl River, N.Y., 0.1 ml, 50 U), and mumps antigen (Lyovac-Mumpsvax, Merck, Sharp and Dohme, West Point, Pa., 0.1 ml, 1/20 dilution of stock solution). The average diameter of 2 right-angle measurements in mm of induration was recorded at 24 and 48 hours.

Thirty-five patients, 21 males and 14 females, with acute leukemia were studied. Twenty-five had acute myelogenous leukemia (AML), and ten had acute lymphoblastic leukemia (ALL). Their median age was 28 years (range 15–71 yr). Their various therapeutic regimens are listed in table 1. All regimens consisted of 5 days of chemotherapy, with intervals off therapy of at least 9 days. The patients were studied as soon as their peripheral blood was free of leukemic cells, just before a course of chemotherapy and at least 5 days after the completion of the previous course. The patients were studied before the second or third course of remission induction therapy and again during the remission maintenance therapy. Of the 35 patients, 27 had not been treated with chemotherapy previously while 8 had had relapses from previous chemotherapeutic regimens. The criteria for remission were described in (23).

RESULTS

A total of 19 of 24 patients with AML and 6 of 11 with ALL had a positive blastogenic response (SI ≥ 2) to autologous leukemia cells in either autologous and/or allogeneic serum. Correlation between the degree of blastogenic response to leukemia cells and the clinical response is shown in table 2. Of 20 patients with an SI >3, 17 (85%) achieved remission. In contrast, only 60% of patients with an SI <3 achieved a clinical remission. All 9 patients with an SI >10 had a clinical remission. Most patients with high SI's had AML. The remission duration also correlated with the relapse rate. Thus, 78% of patients with an SI <3 who achieved remission subsequently had relapses. Their median duration of remission was only 19 weeks. In contrast, only 24% of patients with an SI >3 have had relapses, and none of the patients with an SI >10 have had relapses. Their median duration of remission was >21 weeks. A total of 6 of the 8 ALL patients achieving remission have had relapses in contrast to only 5 of the 18 with AML.

TABLE 1.—*Chemotherapy regimens*

Number of patients treated	Regimen code	Drug (letter code)	Period during course when drug administered (days)	Average dose/ day (mg/M^2)	Schedule* (interval in days)
13	DOAP	Daunomycin (D)	1	60.0	14
		Vincristine (O)	1	2.0†	
		Ara-C (A)	1–5	100.0	
		Prednisone (P)	1–5	200.0†	
9	COAP	Cyclophosphamide (C)	1–5	100.0	14
		Vincristine (O)	1	2.0†	
		Ara-C (A)	1–5	100.0	
		Prednisone (P)	1–5	200.0†	
8	OAP	Vincristine (O)	1–5	100.0	14
		Ara-C (A)	1–5	200.0	
		Prednisone (P)	1–5	200.0†	
2	Guanazole	Guanazole	1–5	25‡	14
2	A-T	Ara-C (A)	1–5	100.0	14
		Thioguanine (T)	2–4	130.0	
1	POMP	6-Mercaptopurine (P)	1–5	500.0	14
		Vincristine (O)	1	2.0†	
		Methotrexate (M)	1–5	7.5	
		Prednisone (P)	1–5	200.0†	

*From day 1 of 1 course to day 1 of next course.
†Total dose/patient/day (in mg).
‡g/M^2.

TABLE 2.—*Correlation between degree of blastogenic response to leukemia cells and clinical response*

	SI<3*	SI>3	SI>5	SI>10
Number remission, Number tested	AML 5/8	13/16	12/13	8/8
	ALL 4/7	4/4	3/3	1/1
	Total 9/15 (60%)	17/20 (85%)	15/16 (94%)	9/9 (100%)
Number relapses, Number remission	AML 3/5	2/13	2/12	0/8
	ALL 4/4	2/4	1/3	0/1
	Total 7/9 (78%)	4/17 (24%)	3/15 (20%)	0/9 (0%)
Median duration† of remission (wk)	19	20+	20+	21+

*Maximum SI in either autologous or allogeneic serum.
†Difference between SI<3, SI>3, $P=0.02$ (Wilcoxin). Difference between SI<3, SI>10, $P=0.002$ (Wilcoxin).

The positive blastogenic response of 9 of the 24 positive patients was partially or completely abrogated when the cultures contained the patient's own serum instead of allogeneic serum (table 3). Eight of these patients had AML.

TABLE 3.—*Correlation between membrane immunofluorescence, blastogenic response, and serum-inhibitory effect*

Blastogenic response (SI>2)	Serum inhibition	Membrane immunofluorescence in types of leukemia			
		AML		ALL	
		Yes	No	Yes	No
Yes	Yes	7*	1	0	1
Yes	No	1	10	0	4
No	No	0	5	0	5
Total		8	16	0	10

*Difference between immunofluorescence in group with serum inhibition and other 2 groups without inhibition highly significant (chi-square-P value <0.005).

Of the 35 patients, 34 were studied for direct membrane immunofluorescence with leukemia cells (table 3). The cells of 8 of 24 patients with AML, but 0 of 10 patients with ALL, demonstrated immunofluorescence with anti-IgG antisera when the cells were incubated either in McCoy's medium and/or the patients' own sera. Morphologically the fluorescence staining appeared as large, nonuniform clumps on the surface of the leukemia cells.

The correlation between the blastogenic response, its serum inhibition, and the presence of membrane immunofluorescence is also shown in table 3. Of the 9 patients with inhibition of blastogenesis by their own serum, 7 had positive immunofluorescence with anti-IgG antisera. In contrast, only 1 of the 15 patients with a positive blastogenic response who did not have a serum-inhibitory effect had a positive immunofluorescence. Finally, cells from patients without a blastogenic response to leukemia cells failed to show this immunofluorescence reaction.

The laboratory and clinical data on the 10 patients, 6 males and 4 females, with the serum-inhibitory effect and/or positive membrane immunofluorescence are shown in table 4. All but patient #9 had AML. Of the 7 patients with positive blastogenesis, serum inhibition, and positive immunofluorescence, 6 achieved a complete clinical remission with chemotherapy. Four of these patients have been studied serially, and the inhibitory effect has been constant in three.

The percentage of fluorescence cells did not increase after the cells were incubated in the patients' own sera compared to incubation with McCoy's media (except for patient #7). Fifty percent of cells of 2 patients (#6 and 10) also reacted with anti-IgM and IgA antisera.

Of the 9 patients with inhibitory effect, 6 had complete abrogation of the blastogenic response when the leukemia cells were incubated in autologous serum, and the inhibition >80% in the other 3 (#3, 4, and 8). In contrast, only 4 of these 9 patients showed significant inhibition of the response to PHA by autologous serum, and in 2 the inhibition was <70%. Thus, the inhibition of blastogenic response to leukemia cells was >80% in 9 of 9, but that of PHA response was >80% in only 2 of 9.

The response of normal allogeneic donor leukocytes to the leukemia cells of the 10 patients with serum inhibition and/or positive immunofluorescence is shown in table 4. Cul-

TABLE 4.—*Patients with serum-inhibitory effect and position immunofluorescence*

Test of clinical or immunologic response	Parameter of evaluation	Source of serum	Patient No. 1	2	3	4	5	6	7	8	9	10
Immunofluorescence	Positive (IgG) (%)	None	20	40	50	50	25	50	5	<5	<5	50
		AB+	20	ND*	ND	48	19	48	23	<5	<5	50
		Autologous	20	40	50	50	15	50	20	<5	<5	50
Blastogenic response of patients' lymphocytes	Tumor cells, cpm†	Autologous	0.0	0.0	18.7	0.7	0.0	0.0	0.0	6.5	0.0	5.1
		Allogeneic	120.0	4.6	23.3	5.1	1.7	1.2	1.1	19.5	0.8	2.6
	Tumor cells, SI	Autologous	0.5	0.5	27	2.4	1.0	0.9	0.8	5.3	0.7	5.9
		Allogeneic	1250	27	517	14.8	3.1	2.5	5.4	38.0	2.4	3.2
	PHA, cpm	Autologous	63.0	48.4	53.6	31.0	57.8	40.2	107.0	50.4	5.6	29.8
		Allogeneic	ND	55.1	64.4	58.5	107.0	41.7	ND	95.6	10.7	35.0
	PHA, SI	Autologous	789	19.8	56.1	103	380	38.8	548	34.5	7.5	35.3
		Allogeneic	ND	23.2	771.0	164	200	107.0	ND	383.0	22.2	43.6
Therapeutic response	Complete remission		Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes
Blastogenic response of allogeneic normal lymphocytes	Tumor cells, cpm	Patient	0.0	4.2	28.8	0.0	16.1	40.5	ND	ND	13.1	24.8
		Normal	38.1	41.7	57.7	3.7	26.3	33.6	7.9	58.3	20.2	11.6
	Tumor cells, SI	Patient	0.9	5.7	9.7	0.1	47	41	ND	ND	4	24
		Normal	376	47.3	18.5	2.4	77	18	15.2	58.0	5.6	32

*ND = not done.
†per 10^6 lymphocytes × 10^3.

tures containing allogeneic lymphocytes were incubated in both the patient's serum and the donor (allogeneic) serum. In 3 instances, the response of donor allogeneic lymphocytes was significantly inhibited with the patient's serum as compared to the donor serum. Thus, in at least 3 instances, inhibitory serum blocked both autologous and allogeneic lymphocyte responsiveness to leukemia cells.

The sera of 5 of the 25 patients with positive blastogenic responses showed a facilitory effect on the blastogenic response to autologous leukemia cells (table 5). There was a significant increase in the tumor response of the patients' lymphocytes cultured in autologous serum compared to allogeneic serum. Of the 5 patients, 4 had AML and all achieved clinical remission. Three also had a significant facilitation of the response to PHA in autologous serum. Two of these and an additional patient also had an increased response to SLO in autologous serum. Thus, 4 or 5 had an increased response to either PHA and/or SLO in autologous serum, and therefore the facilitory effect was nonspecific.

The correlation between general immunocompetence as measured by the response to PHA and SLO in lymphocyte response to autologous leukemia cells is shown in table 6. The overall median response to PHA in cpm was somewhat lower in the group with the SI <3, compared to the group with an SI >3. The SI of the former group was clearly lower than the latter. Of greater interest was the response to SLO in these groups of patients. Those with an SI to autologous leukemia cells >3 had a strikingly superior response to SLO than those with a response <3. A similar pattern was noted for the SI to SLO. Despite this difference, only 1 of 15 patients with an SI to autologous leukemia cells <3 had a subnormal response to PHA (cpm $<20{,}000$) (*24*).

However, 7 of 15 patients with an SI <3 had a subnormal response to SLO (cpm <2000) (*24*), compared to 5 of 20 with an SI >3. Thus the proportion of patients considered immunoincompetent was only slightly greater in the group with a poor response to autologous leukemia cells. The overall response by AML and ALL patients to PHA was comparable (median net cpm 60×10^3 and 67×10^3, respectively). Patients with AML had a somewhat more vigorous response to SLO than those

TABLE 5.—*Blastogenic response to autologous leukemia cells—serum-facilitory effect*

Patient No.	Diagnosis	cpm×10³		SI		Clinical response
		Autologous	Allogeneic	Autologous	Allogeneic	
1	AML	3.6	1.08	5.7	1.9	R
2	AML	9.5	.09	59.7	0	R
3	AML	4.5	0	80.9	0	R
4	AML	1.82	0	12.9	0	R
5	ALL	3.3	.08	10.0	1.4	R
Median		3.6	.08	12.9	0	

TABLE 6.—*Correlation between blastogenic response to leukemia cells, PHA, and SLO*

Blastogenic response to leukemia cells	SI	<3	>3	>5	>10
Blastogenic response to PHA	Net cpm × 10^3	62.0* (11.0–114.0)	84.0 (13–148)	69.0 (13–145)	55.0 (13–145)
	SI†	139 (4.7–500)	322 (13–1116)	250 (13–1116)	464 (13–1116)
Blastogenic response to SLO	Net cpm × 10^3	2.3 (0–46.0)	13.2 (0–98)	13.5 (0–98)	17.7 (0–98)
	SI‡	3.6 (1.0–162)	17.5 (1.5–843)	14.7 (1.5–843)	16.0 (1.5–843)

*All values are medians with ranges in parentheses. Values represent maximum response in autologous or allogeneic serum.
†Difference in PHA response between SI<3 and SI>3 groups $P=0.05$.
‡Difference in SLO response between SI<3 and SI>3 groups $P=0.013$.

with ALL (8.5×10^3 compared to 3.7×10^3 cpm, respectively).

Nineteen patients achieving a clinical remission had repeat studies during the remission maintenance phase of their treatment. These results are shown in table 7. Of the 11 patients who had a positive blastogenic response in remission, 10 (91%) have continued in remission. In contrast, 7 of 8 patients who had a negative response to leukemia cells during remission have had relapses. Two of these patients were positive initially. Of the 6 patients whose negative (SI<2) blastogenic responses persisted, 5 have had relapses.

The overall blastogenic responses to leukemia cells by patients' lymphocytes and allogeneic normal lymphocytes cultured concurrently are shown in table 8. The median response of AML patients to their own leukemia cells was considerably greater than that of ALL patients to their own cells. Similarly, normal subject lymphocytes responded to a significantly greater degree to AML cells than to ALL cells.

Table 9 summarizes the patients' lymphocyte blastogenic responses to soluble antigen and autologous leukemia cells. Five patients had AML, one had ALL, and one had blast crisis of chronic myelogenous leukemia. Four of the five patients with AML and the patient with ALL had significant blastogenic response to autologous leukemia cells. All 5 of these paitents as well as the patient with blast crisis had significant blastogenic responses to soluble antigen. The response to soluble antigen was greater than the response to cells in patients #2 and #7. In contrast, the response to intact leukemia cells was greater in patients #3 and #4. Of the 5 patients achieving remission, 4 re-

TABLE 7.—*Serial blastogenic response to leukemia cells in patients achieving remission*

Change in blastogenic response		Change in clinical status	
		Remission → remission	Remission → relapse
Initial study	Follow-up study		
Positive*	→ Positive	9	1
Negative	→ Positive	1	0
Positive	→ Negative	0	2
Negative	→ Negative	1	5

*Positive = SI≥2. Negative = SI≤2.

TABLE 8.—*Blastogenic response to leukemia cells*

	Net cpm × 10^3		SI		
	AML	ALL	AML	ALL	*P*
Patients' lymphocytes	4.6* (0–120.0)	0.449 (0–3.35)	5.4 (0.9–1250)	2.6 (0.7–12.1)	0.06
Normal lymphocytes	34.9 (0.937–104.0)	11.1 (2.6–76.2)	35.9 (1.6–579.0)	17.7 (2.9–113)	0.35

*All values are medians with ranges in parentheses.

TABLE 9.—*Blastogenic response to autologous leukemia cells (LC) and soluble leukemia cell antigen**

Cell and antigen No.	Net cpm × 10^3		SI		Effect of therapy
	LC	Antigen	LC	Antigen	
1	1.7	2.4	3.1	5.4	Remission
2	5.9	21.8	10.8	39.6	Remission
3	9.2	1.0	30.5	4.4	Remission
4	19.5	1.6	38.0	6.4	Remission
5	0	0.62	0.8	1.1	Remission
6	0.72	.56	3.6	2.8	Failure
7	0	.64	0.9	3.1	Failure

*Maximum response in autologous or allogeneic serum.

sponded to antigen with an SI >4. In contrast, the 2 patients who failed to achieve a bone marrow remission had SI's <4.

The blastogenic response of normal allogeneic donor lymphocytes to leukemia cells and soluble antigen is shown in table 10. Six of the seven leukemic cell preparations elicited a vigorous blastogenic response in vitro. In contrast, only one of the soluble antigen preparations (No. 1) induced a significant, but weak, response.

TABLE 10.—*Blastogenic response of normal human lymphocytes to allogeneic leukemia cells (LC) and soluble leukemia cell antigen*

Cell and antigen No.	Net cpm × 10^3		SI	
	LC	Antigen	LC	Antigen
1	26.0	0.53	75.4	2.7
2	30.0	0.08	60.0	1.2
3	18.0	0	12.5	0.1
4	65.0	0	65.0	0.7
5	25.0	0	52.0	0.7
6	0.17	0.18	1.6	1.6
7	34.0	0.10	66.0	1.2

Although dose-response relationships were noted in these studies, good stimulation was noted with doses as low as 10–100 μg/ml. Because of the limited availability of the antigen, doses >1000 μg/ml of culture were not used. In at least 2 patients, the maximum stimulating dose of antigen may not have been used, since the dose-response curve was still rising at 1000 μg/ml.

None of the 34 patients demonstrated positive delayed-hypersensitivity reactions to intact leukemia cells with doses as high as 10^6/0.1 ml. Two of the seven patients (#1 and #3) skin-tested with antigen reacted with a positive skin test. The positive delayed-hypersensitivity skin reaction was confirmed by punch biopsies. Of the 5 patients who did not demonstrate delayed hypersensitivity to the soluble antigen, however, 4 were anergic to the battery of 5 established skin test antigens at the time of testing.

DISCUSSION

To understand more completely the patients' immune responses to autologous tumor, one must consider both the cell-associated and humoral aspects of tumor immunity. The series of studies in acute leukemia reported here were designed to determine the interrelationship between lymphocyte blastogenic responses to leukemia cells, serum effects on these responses, and the presence or absence of immunoglobulin on the leukemia cells. In addition to the knowledge gained, this type of combined approach should form a background for the design of rational immunotherapy programs in acute leukemia and in other malignancies.

Previous work from this laboratory demonstrated that immunocompetence is associated with a favorable prognosis in patients with acute leukemia (*24*). The present study identified several new features that may be of relevance to the prognosis of these patients. In addition to confirming previous observations that the lymphocytes of most patients with acute leukemia show a blastogenic response to their own leukemia cells (*5, 6, 8*), we have identified a strong correlation between the intensity of lymphocyte responsiveness to autologous leukemia cells early in therapy and the likelihood for achieving a subsequent remission. Even more significant, the duration of remission is superior in patients with vigorous responses to their leukemia cells. Some previous studies failed to identify this correlation (*8*). However, Oren and Herberman (*4*) found a correlation between the positive response to skin tests with autologous leukemia membranes and clinical remissions in ALL patients.

It is interesting to speculate that the strong immune response to leukemia cells may augment the chemotherapeutic effects and be a factor in the remission induction process. Similar speculations have been offered as an explanation for the dramatic responses seen with chemotherapy in patients with Burkitt's lymphoma (*25*) and choriocarcinoma (*26*).

This study has also shown the importance of serial studies of tumor immunity in patients with leukemia. The persistence of a positive blastogenic response in remission or a change from negative to positive was associated with a good prognosis. In contrast, patients who repeatedly had negative blastogenic response to their leukemia cells during remission or who had gone from positive to negative had early relapses. Therefore, these techniques should be used to evaluate patients on long-term chemo-

therapy and be used to guide future immunotherapy trials in acute leukemia.

Earlier studies of humoral responses to human leukemia cells have only rarely detected immunoglobulin on the cell surfaces. The current demonstration that positive membrane immunofluorescence occurred as frequently when the cells were incubated in McCoy's media or the patients' own sera suggests that immunoglobulin is already coated on the cells. Whether the cells themselves are producing the IgG immunoglobulin—as Burkitt lymphoma cells produce IgM (*27*)—or the cells are being coated from the outside—as described for the IgG on some Burkitt cells (*28*)—cannot be determined by these studies.

The idea that the positive fluorescence represents bound immunoglobulin in our group of patients is substantiated by the morphologic appearance of the immunoglobulin on the tumor cells. Immunoglobulin receptors on cells of B origin show either a fine, uniform ring pattern of fluorescence or cap formation (*29*). In contrast, the immunoglobulin on the leukemia cells in this study appears as nonuniform, larger clumps of staining, characteristic of bound immunoglobulin.

Only patients with AML had evidence of bound immunoglobulin. Thus patients with ALL either do not make such antibody or perhaps their leukemia cells are not very immunogenic. The greater autologous blastogenic response to AML cells compared to ALL cells also suggests this possibility. The fact that ALL cells do not appear to produce surface immunoglobulin as do tumor cells from other lymphoid tumors, such as chronic lymphocytic leukemia (*30*) and malignant lymphoma (*31*), suggests that these cells may be of thymic origin, may not have differentiated sufficiently to produce immunoglobulin, or may not be of lymphoid origin.

Patients with cancer may have factors in their serum which inhibit cell-associated responses to various mitogens and tumor cells (*10*). The Hellströms demonstrated a tumor-specific blocking or enhancing antibody in the serum of many patients with progressively growing solid tumors (*11*). Mavligit et al. (*32*) and Vánky et al. (*33*) demonstrated serum inhibition of blastogenic reactivity to tumor cells in patients with solid tumors. Initial studies by the Hellströms suggested that the blocking factor was a 7S immunoglobulin. Recently, Sjögren et al. (*34*) suggested that the blocking factor may be tumor antigen-antibody complex. Although our present study showed a strong correlation between the presence of immunoglobulin on leukemia cells and the blocking of the blastogenic response, it does not clarify whether antibody, antigen-antibody complexes, or perhaps other factors are involved with the inhibitory phenomenon.

In the present study, the presence of immunoglobulin on leukemia cells and the presence of an inhibitory effect on the blastogenic response were correlated with a good prognosis. Six of the seven patients with this constellation of findings achieved a clinical remission. Five of these patients remained in remission from 3 to 12 months. The serum-inhibitory effect has been consistent throughout the clinical course in 75% of patients tested serially.

The demonstration of a serum-facilitory effect in 5 patients with acute leukemia demonstrates that the effect of serum factors on in vitro cell-associated immunity is complex and requires careful investigation in individual patients with all types of malignancies. The pathogenesis of this effect is unclear, but it appears to be associated with a relatively good prognosis. None of these patients had demonstrable immunoglobulin on their leukemia cell surfaces. The possibility exists that there is circulating antigen, or antigen-antibody complexes, in autologous serum which evokes an additive blastogenic effect in vitro Al-Sarraf et al. (*35*) reported that lymphocyte response to PHA is reduced when incubation is done in allogeneic serum rather than autologous serum. Therefore, we may be seeing inhibition by allogeneic serum rather than autologous facilitation. At any rate, the evaluation of serum factors in all studies of cell-mediated immunity is of great importance in attempting to understand the immune response to human tumors.

The in vitro lymphocyte responses to PHA and SLO do not always parallel the patient's response to autologous leukemia cells. As a group, patients not responding to their own

leukemia cells tended to have a lower response to PHA than patients with a positive blastogenic response to leukemia cells. Only one patient in the former group could be considered immunoincompetent by the response to PHA. However, several patients nonresponsive to leukemia cells had poor responses to the antigen SLO. Since macrophages are required for the in vitro lymphocyte responsiveness to antigens (*36*), one could speculate that impaired processing of antigen by macrophages is one reason for the lack of tumor cell responsiveness in some patients. Against this is the fact that several patients with vigorous responses to leukemia cells also had poor SLO responsiveness. Hence, the evaluation of general immunocompetence does not invariably parallel the response to autologous leukemia cells.

Another factor which may be as important in the tumor responsiveness of patients is the antigenicity of leukemia cells. Our data strongly suggest that patients with AML have a more intense immune response to their own tumor cells than do patients with ALL. As a group, there was no difference between AML and ALL patients in their response to PHA and only a slight difference in their response to SLO. The evidence that AML may be associated with more intense immune response is further substantiated by the superior response of allogeneic normal lymphocytes to AML cells compared to ALL cells. There may be antigen deletion of some ALL cells. This has been suspected in a patient with malignant lymphoma (*37*) and in some animal leukemias (*38*). Alternatively, there may be antigen masking by sialic acid residues (*39*).

Our data indicate that antigen extracted from human leukemia cells by 3M KCl can be used to stimulate autologous lymphocytes in lymphocyte cultures. The results correspond quite well with those obtained with the lymphocyte response to autologous leukemia cells.

Except for one report to the contrary (*40*), most workers have reported that soluble histocompatible antigens can stimulate lymphocyte blastogenesis only after prior sensitization (*41*, *42*). Only one of the normal donors in the current study responded to the allogeneic soluble antigen preparations, and even that response was very weak, whereas 6 of the 7 leukemia patients responded to their autologous antigen. Thus, the data strongly support the idea that this lymphocyte stimulation indicates prior sensitization to the antigen and represents specific tumor immunity. A similar observation has recently been made in a patient with colon carcinoma (*43*). It was suggested that peripheral blood and lymphocytes can undergo primary in vitro blastogenic responses to foreign antigens only if the latter are cell associated. If they are solubilized, a response will occur if the subject is immune. Whether presensitization is required to respond to cell-associated leukemia antigen is unclear (*44–46*).

Thus, the study has demonstrated the usefulness of soluble antigen in the study of tumor immunity in patients with leukemia (*47*). Hopefully, the use of soluble antigen will identify subjects previously sensitized with leukemia-associated antigens and may help in the search for common antigens in leukemia cells. In addition, as the preliminary observations with skin testing suggested, such antigen preparations should be useful in the other assays of cell-mediated immunity, such as migration inhibition and delayed hypersensitivity.

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Cell-Mediated Immunity to Human Solid Tumors: In Vitro Detection by Lymphocyte Blastogenic Responses to Cell-Associated and Solubilized Tumor Antigens [1, 2]

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SUMMARY—Cell-mediated immunity to human solid tumors could be demonstrated in vitro by the lymphocyte blastogenesis method. Blastogenic responses to unseparated autochthonous tumor cells were positive in 19 of 29 patients. The intensity of response was dose related, and the maximum response required a tumor cell-lymphocyte ratio of 1:1. The intensity of response was also correlated inversely with the extent of disease. The effect of serum from cancer patients on such responses in vitro was complex. It could be both inhibitory or facilitory and did not correlate with the extent of the disease. Separation of viable from nonviable tumor cells by specific density solution (Ficoll-Hypaque), and their respective treatment with irradiation, mitomycin C, or nothing, showed that viable tumor cells were superior to nonviable cells and irradiation was superior to mitomycin C in inducing lymphocyte blastogenesis. Irradiated viable tumor cells appeared to be the material of choice in the study of lymphocyte blastogenic response to cell-associated tumor antigen. Soluble antigen, extracted with 3M KCl induced vigorous blastogenic responses in autologous lymphocytes, but no such stimulation occurred among normal allogeneic lymphocytes. An immunologic difference between cell-associated antigens and their soluble form was hypothesized.—Natl Cancer Inst Monogr 37: 167–176, 1973.

STUDIES in the past 7 years proved the presence of tumor-distinctive antigens in most human solid tumors (*1–3*). More importance was attached to cell-mediated immunity than to humoral immunity in respect to the host's immune defense against solid tumors, since transplantation experiments showed that effective tumor immunity is based on lymphocytic mechanisms similar to those of homograft rejection (*4*).

In vitro lymphocyte blastogenic responses to tumor cells were first studied by Stjernswärd et al. (*5–7*) using techniques similar to those of the mixed leukocyte reaction (*8*). A positive blastogenic response to autochthonous tumor cells could be interpreted as a primary cellular recognition of nonself, tumor cell-surface antigens. On the other hand, sensitization to these antigens should occur in vivo, and subsequent blastogenic response of circulating memory cells to tumor cells in vitro could reflect established tumor immunity. Two observations tend to support the latter view: 1) Soluble tumor antigens obtained from a cystic melanoma induced blastogenic responses among autologous lymphocytes and lymphocytes from other patients with melanoma but not among lymphocytes from normal subjects (*9*). 2) The intensity of lymphocyte blastogenic responses to leukemia cells can be augmented temporarily

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by active immunization with irradiated leukemia cells (*10*).

We present our current experience with the lymphocyte blastogenesis assay for tumor immunity. We confirm previous reports that freshly obtained tumor cells from a variety of solid tumors can stimulate blastogenic responses among autologous lymphocytes from most patients (*5–7*). For the optimal study of tumor immunity with this system, viable tumor cells are superior to nonviable cells, and irradiation of tumor cells is superior to their treatment with mitomycin C. The degree of blastogenic response correlates with the stage of disease, and a vigorous response indicates a good prognosis. Serum effects were complex, and patients' sera had both suppressive and facilitory effects on the blastogenic response to tumor cells. Finally we present preliminary evidence that human tumor-associated antigen can be separated by salt extraction and effectively used to detect cell-mediated tumor immunity.

MATERIALS AND METHODS

Patients.—Of 42 patients, age 18–81 years, 12 had melanoma, 8 had carcinoma of the colon, 6 had sarcoma, 4 had lymphoma, 3 had squamous cell carcinoma of the lung, 2 had gastric carcinoma, 2 had carcinoma of the ovary, 2 had basal cell carcinoma, and 1 each had carcinoma of the breast, hypernephroma, and carcinoma of unknown origin. Before any treatment, 15 patients were studied; 17 patients had previous surgery, 8 had received previous chemotherapy (but not within 4 weeks before study), and 7 had been given previous radiotherapy (but not within 3 months before study). Patients differed in their stage of disease. Localized disease was defined as primary tumor, either without spread or with spread, but not beyond the regional lymph nodes. Widespread disease was defined as spread beyond the regional lymph nodes.

Tumor cells and tumor antigens.—For preparation of single tumor cell suspensions from fresh tumor biopsies, cells were teased, scraped, and sieved through a 60-mesh screen into medium 199 (Gibco Laboratories, Grand Island, N.Y.) containing 1 U penicillin and 1 μg streptomycin/ml. Cells were washed once and counted; their viability was determined by exclusion of trypan blue (*11*). Samples for bacteriologic cultures were taken frequently. Unseparated (viable from nonviable) tumor cells were either irradiated with 4000 R or remained untreated before use as lymphocyte stimulants.

For separation of viable from nonviable tumor cells, a solution of specific density made up of Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden) and Hypaque (Winthrop Laboratories, New York, N.Y.) was prepared as follows: 24 parts of 9% Ficoll and 10 parts of 33.9% Hypaque were thoroughly mixed to give a solution of a final density of 1.08 g/ml. This mixture was sterilized by autoclaving. Approximately 10^8 tumor cells in 2 ml of medium 199 were carefully layered on top of 10 ml specific-density solution in a 25×150-mm screw-cap centrifuge tube. This was centrifuged at 1500×g for 15 minutes. The cells in the fraction floating between the medium 199 and the Hypaque-Ficoll solution and the cells in the pellet at the bottom of the tube were removed separately and washed twice in minimal essential medium (MEM, Gibco Laboratories); each fraction was separated into 3 parts: 1) Tumor cells were treated with mitomycin C (Bristol Laboratories, Syracuse, N.Y.) as described in (*12*); 2) they were irradiated with 4000 R; and 3) they remained untreated.

For extraction of tumor antigens, a single cell suspension was prepared as described above. Red blood cells were lysed by Tris-buffered ammonium chloride for 15 minutes as in (*13*), and 5×10^9 tumor cells were mixed with 50 ml of 3M KCl made up in phosphate-buffered saline (PBS) at pH 7.2 as in (*14*). After equilibration on a rocker at 4° C for 16 hours, the contents of the tube were centrifuged at 0° C at 40,000×g for 60 minutes. The slightly viscous supernatant was dialyzed against 20 volumes of deionized water for 1 hour. This was followed by dialysis against 20 volumes of PBS for 24 hours with change of solution every 8 hours. The dialyzed extract was centrifuged at 0° C at 40,000×g for 30 minutes and the supernatant was concentrated by pervaporation. The concentrated extract was mixed with an equal volume of 4M ammonium sulfate. The precipitate was centri-

fuged at 40,000×g at 0° C for 30 minutes. The supernatant was discarded, and the precipitate redissolved in 5 ml PBS, pH 7.2. It was finally dialyzed against PBS for 4 hours. The protein concentration was estimated by measurement of optical density at a wavelength of 280 nm (OD 280). The final preparation was sterilized by passage through a 0.45μ Millipore filter (Millipore Corp., Bedford, Mass.). It was stored at 4° C with frequent checks for bacterial contamination.

In selected cases, normal cellular organs were obtained from cancer patients. They included an adrenal gland from a patient with carcinoma of the breast, an ovary from another patient with carcinoma of the breast, a kidney from a patient with transitional carcinoma of the ureter, and a testis from a patient with carcinoma of the prostate. In all, the gross pathologic examination and the histologic sections revealed no evidence of tumor involvement. Single cell suspensions were prepared as described for tumor cells. Cells were either irradiated or remained untreated before use as lymphocyte stimulants.

Lymphocyte cultures.—For preparation of lymphocytes, venous blood was drawn on the day of surgical removal of tumor, but before sedation, anesthesia, or surgery. It was defibrinated by being swirled with glass beads for 10 minutes, and the red blood cells were sedimented with dextran as described (*15*). The leukocyte-rich serum was collected, and the white blood cell and differential counts were determined. For each experiment, venous blood was also drawn from a normal, healthy subject, and lymphocytes were prepared in an identical manner. Both patients' and normal subjects' lymphocytes were centrifuged at 140×g for 10 minutes at room temperature (International Centrifuge, Model UV). Serum was removed and heat inactivated at 56° C for 30 minutes. The lymphocytes were washed with MEM.

Four groups of lymphocyte cultures were set up in 16×125-mm screw-cap centrifuge tubes. Each tube contained 10^6 lymphocytes, 2 ml MEM, and 1 ml heat-inactivated serum. Cancer patients' lymphocytes were cultured in both autologous and allogeneic sera (the first and second groups), and normal subjects' lymphocytes were likewise cultured in their own serum and also in serum from a cancer patient (third and fourth groups).

In addition to unstimulated control cultures, each of these 4 groups was stimulated with phytohemagglutinin M (PHA, Difco Laboratories, Detroit, Mich.), allogeneic leukocytes (MLC) irradiated with 4000 R (usually exchanged between the cancer patient and the normal subject studied on the same day), and with autologous, irradiated leukocytes.

Stimulation of lymphocyte cultures with tumor material was conducted in 3 ways: 1) with unseparated tumor cells; in this case, 10^4–2×10^6 irradiated or untreated tumor cells in half-log dose intervals; 2) with separated tumor cells; in this case, 5×10^4–1×10^6 viable and nonviable tumor cells treated separately with irradiation, mitomycin C, or nothing, again in half-log dose intervals; and 3) with extracted tumor antigens, in a dose range of 20–1000 μg/ml of culture. Tumor cells, in the above preparations, were always cultured simultaneously in identical doses and after identical treatments but without lymphocytes.

In the selected cases in which normal tissue from a cancer patient was available, 10^6 autologous lymphocytes were stimulated with 10^4–1×10^6 normal tissue cells. Simultaneously, normal tissue cells were also cultured in identical doses but without lymphocytes.

All cultures were incubated at 37° C in an atmosphere of 5% CO_2 in air. Cultures containing tumor cells or normal tissue cells were incubated 7 days and 2 μCi ^{3}H-thymidine (specific activity 1.9/mM, Schwarz-Mann, Orangeburg, N.Y.) was added to each culture 3 hours before they were harvested as described in (*15*). Cultures stimulated with extracted tumor antigen were incubated under identical conditions but only for 3–5 days, and 1 μCi of the isotope was added to each of the cultures 8 hours before they were harvested. Blastogenesis was taken as the incorporation of ^{3}H-thymidine in the acid-insoluble fraction, measured by liquid scintillation counting and expressed as counts per minute (cpm) per 10^6 lymphocytes. The true blastogenic response to tumor was defined as the cpm of cultures containing lymphocytes with tumor cells minus the cpm of cultures containing tumor cells alone. The stimulation

index (SI) was defined as the true blastogenic response divided by the cpm of cultures containing unstimulated lymphocytes. An SI ≥ 3.0 was considered significant stimulation by tumor cells, the reasons for which will be discussed below.

Significant serum inhibition of the blastogenic response was defined as a $\geq 50\%$ drop in the SI in one serum compared to the other. Significant facilitation of blastogenesis was defined as $\geq 100\%$ rise in the SI in one serum compared to the other. Data were analyzed statistically with the Wilcoxon signed rank test (*16*).

RESULTS

The blastogenic responses to autologous leukocytes were not significantly different from those of the unstimulated control cultures. Lymphocyte blastogenic responses to optimal doses of normal autologous tissue cells in the selected cases so studied never exceeded an SI of 3.0. Accordingly, positive or significant lymphocyte blastogenic responses to optimal dose of autochthonous tumor cells were defined as an SI ≥ 3.0. This was observed in 19 of 29 patients studied with unseparated tumor cells (table 1). The median SI among these responders was 13.6 and corresponded to a median true blastogenic response of 3405 cpm. In contrast, lymphocytes from 24 of 27 normal subjects responded to optimal doses of these allogeneic tumor cells with an SI ≥ 3.0. The median SI among these responders was 20.1 and corresponded to a median true blastogenic response of 9475 cpm. The difference in blastogenic response to tumor between patients and normal subjects was statistically significant at the 5% level and probably represented the response of the latter to histoincompatible MLC antigens.

The blastogenic response to tumor cells was dose related (table 2). A median SI >3.0 was observed among cancer patients' lymphocytes when $\geq 10^5$ tumor cells were used for stimulation. A maximum SI of 6.5 among cancer patients' lymphocytes was achieved with 10^6 tumor cells. This was equivalent to a tumor cell-lymphocyte ratio of 1:1. An excess of tumor cells with a tumor cell-lymphocyte ratio of 2:1 not only failed to augment stimulation but also inhibited it somewhat. Stimulation of normal subjects' lymphocytes to an index >3.0 was also achieved with the use of $\geq 10^5$ tumor cells and reached its maximum (12.1) when 5×10^5 tumor cells were used. This was equivalent to a tumor cell-lymphocyte ratio of 1:2. Again, an increase of the tumor cell-lymphocyte ratio to 1:1 or 2:1 did not augment the blastogenic response. Although the dose-response relationship was similar in both patients and normal subjects, the normal subjects showed considerably greater responses to each tumor cell dose above the critical level of 10^5 tumor cells per culture.

The relationship between the stage of disease and the blastogenic response to optimal doses of autochthonous tumor cells and to PHA is

TABLE 1.—*Lymphocyte blastogenic responses to optimal doses of unseparated tumor cells*

Responder cells	SI > 3.0 observed/studied		Median SI among responders	Median cpm among responders
Cancer patients' lymphocytes	19/29	(65.5%)	13.6	3405
Normal donors' lymphocytes	24/27	(88.8%)	20.1	9475

TABLE 2.—*Lymphocyte blastogenic responses (SI's) to graded doses of unseparated tumor cells*

	Tumor cell dose used for stimulation				
Response	10^4	10^5	5×10^5	10^6	2×10^6
Of cancer patients	2.0	3.6	4.0	6.5	4.4
Of normal subjects	2.2	3.4	12.1	11.5	11.5

shown in table 3. The median SI of the 16 patients with localized disease was 13.2; of the 13 patients with widespread disease, it was only 4.9. This was statistically significant at the 6% level. In contrast, no such relationship was observed after stimulation of lymphocytes with PHA. The median SI in the 16 patients with localized disease was 207, whereas in the 13 patients with widespread disease it was 266.

The effect of heat-inactivated serum from cancer patients on the lymphocyte blastogenic response to optimal doses of tumor cells is shown in table 4. Response was significantly inhibited by autologous serum in 7 and significantly facilitated in 9 of 28 patients so studied. In only a few patients, whose lymphocyte blastogenic responses were either inhibited or facilitated by autologous serum, was the effect tumor specific. Thus in most, the simultaneous responses of the same lymphocytes to PHA and allogeneic leukocytes were modulated by serum in the same direction as the response to the tumor cells. Also observed in some patients was the reverse phenomenon, i.e., the nonspecific inhibitory or facilitory effects of serum on the blastogenic responses to PHA and allogeneic leukocytes in the absence of effect on the response to tumor. Similarly, the blastogenic responses of normal subjects to tumor cells were significantly inhibited by sera from the respective tumor-bearing hosts in 9 and facilitated in 6 of 27 subjects so studied. Again, only in a few normal subjects, whose lymphocyte blastogenic responses were inhibited or facilitated by sera from tumor-bearing hosts, was the effect tumor specific. There was no correlation between any serum effect and the stage of disease or between the effects on normal subjects' compared to patients' responses.

Separation of viable from nonviable tumor cells by means of specific density solution centrifugation (table 5) increased the viable cell population from a median of 16% (2–57%) before separation, to a median of 77% (65–97%) in the floating fraction after separation ($P < 0.001$). The pellet consisted almost exclusively of nonviable cells, with a median purity of 98.5%.

Treatment of viable and nonviable tumor cells with irradiation, mitomycin C, or nothing in 11 patients so studied resulted in populations of tumor cells which induced in their optimal doses the blastogenic responses shown in table 6.

The median blastogenic response to irradiated viable tumor cells was 4608 cpm and exceeded the response to untreated viable tumor cells (4068 cpm) and to viable tumor cells

TABLE 3.—*Blastogenic responses (SI's) of cancer patients' lymphocytes to tumor cells and PHA; relation to stage of disease*

	Tumor cells		PHA	
	Localized disease	Widespread disease	Localized disease	Widespread disease
Number	16	13	16	13
Mean	20.0	8.0	231.0	300.0
SD	20.3	9.6	177.8	223.5
Median	13.2	4.9	207.5	266.4
Range	1.3–62.5	0–34.5	0.7–805.1	0.8–710.7
P	0.06		0.3	

TABLE 4.—*Effect of cancer patients' sera on the blastogenic responses of autologous and normal donors' lymphocytes to optimal doses of tumor cells, to PHA, and to allogeneic leukocytes (MLC)*

	Stimulation with tumor cells		Stimulation with PHA		Stimulation in MLC	
Responder cells	Inhibition	Facilitation	Inhibition	Facilitation	Inhibition	Facilitation
Cancer patients' lymphocytes	7/28*	9/28	7/28	9/28	9/28	10/28
Normal donors' lymphocytes	9/27	6/27	9/26	5/26	7/22	9/22

*Number observed/No. studied.

TABLE 5.—*Separation of viable from nonviable tumor cells on Ficoll-Hypaque specific density solution; 10 experiments*

	Percent viable tumor cells			Viable tumor cells recovered in floating fraction	
	Before separation	Floating fraction	Pellet	Absolute No. ($\times 10^6$)	Percent initial total cell count
Mean	21.6	79.9	7.6	6.9	12.4
Median	16	77	1.5	4.6	6.5
Range	2–57	65–97	1–30	0.2–21	0.5–47
SD	18.3	12.8	10.0	6.9	14.9

TABLE 6.—*Median blastogenic responses and incidence of SI $\geq$3.0 in patients' lymphocytes to optimal doses of autochthonous viable and nonviable tumor cells treated with irradiation (XRT), mitomycin C (mito C), or untreated*

Tumor cells	True blastogenic response cpm/10^6 lymphocytes	SI	Number positive with SI $\geq$3.0/ No. tested (% positive)
Viable, XRT	4608	6.8	9/11 (82)
Viable	4068	4.5	8/11 (73)
Nonviable	4004	5.1	7/11 (64)
Nonviable, XRT	1780	4.7	7/11 (64)
Viable, mito C	1411	2.0	3/10 (30)
Nonviable, mito C	1359	1.7	3/10 (30)

treated with mitomycin C (1411 cpm). The median response to untreated nonviable tumor cells was 4004 cpm, while the responses to nonviable tumor cells treated with irradiation or mitomycin C were 1780 and 1359 cpm, respectively. Thus the efficiencies of the various tumor cell preparations in inducing blastogenesis (in descending order) were: irradiated viable, viable, nonviable, irradiated nonviable, and mitomycin C-treated viable and nonviable tumor cells.

The SI's correlated well with the true blastogenic responses. Thus stimulation with irradiated viable tumor cells resulted in an index of 6.8, whereas stimulation with viable and nonviable tumor cells treated with mitomycin C resulted in indices of 2.0 and 1.7, respectively. The incidence of SI's $\geq$3.0 is also shown in table 6. An incidence of 82% was observed with irradiated viable tumor cells and only 30% with either viable or nonviable tumor cells treated with mitomycin C.

Statistical analysis of the data showed that viable tumor cells, irradiated or untreated, were significantly superior to mitomycin C-treated viable and nonviable tumor cells. Although irradiated viable tumor cells were also superior to irradiated and untreated nonviable tumor cells, the difference was not significant at the 5% level. In addition, nonviable tumor cells, irradiated or untreated, were significantly better than mitomycin C-treated nonviable tumor cells.

The blastogenic responses of the lymphocytes of 2 patients to extracted autochthonous tumor antigen were studied and compared with the responses of the lymphocytes of 2 normal healthy subjects. Maximal blastogenic response to tumor antigen was observed among the lymphocytes of the patients (table 7) when a dose of 100 μg/ml was used. The blastogenic response was partially inhibited by 1 patient's serum. Higher doses of antigen inhibited the blastogenic response. Blastogenic response to tumor antigens was not detected among the normal donors' lymphocytes, despite an adequate response to PHA, to the patients' lymphocytes (MLC), and in the absence of serum-inhibitory effect. Both antigen extracts induced positive skin reactions in the respective patients. These were proved by biopsy to be delayed hypersensitivity.

DISCUSSION

Lymphocyte blastogenic responses to optimal doses and preparations of autochthonous tumor cells can be detected in most patients with solid tumors. To rule out the possibility that

TABLE 7.—*Blastogenic responses (cpm) of patients' and normal donors' lymphocytes to autochthonous, KCl-solubilized tumor antigens, and other stimulants. Numbers in parentheses indicate SI*

Stimulants	Patient 1 (carcinoma of colon)		Normal donor 1		Patient 2 (carcinoma of ovary)	Normal donor 2
	Autologous serum	Normal serum	Autologous serum	Patient 1 serum	Autologous serum	Autologous serum
None	112	111	209	247	197	1,463
PHA	40,468(361)	48,522(437)	149,856(717)	176,990(716)	43,878(222)	78,982(54)
10^6 allogeneic leukocytes	4,570(41)	5,420(49)	8,493(40)	12,172(49)	16,734(85)	16,552(11)
Tumor antigens (μg/ml)						
10	–	–	–	–	954(4.8)	2,320(1.6)
20	–	–	326(1.5)	154(0.6)	–	–
40	–	–	162(0.8)	380(1.5)	–	–
50	–	–	–	–	696(3.5)	1,444(0.9)
60	3,174(28)	6,034(54)	–	–	–	–
80	–	–	228(1.1)	228(0.9)	–	–
100	4,960(44)	14,034(126)	–	–	1,810(9.2)	1,872(1.3)
170	–	–	317(1.5)	668(2.7)	–	–
250	–	–	–	–	1,278(6.5)	2,640(1.8)
330	–	–	299(1.4)	232(0.9)	–	–
500	–	–	–	–	1,058(5.4)	1,908(1.3)
600	162(1.4)	170(1.5)	–	–	–	–
1000	46(0.4)	332(3.0)	–	–	–	–

the response to tumor cells is to nonspecific tissue mitogens or tissue-distinctive antigens (*17*), rather than to tumor-distinctive antigens, we studied the patients' lymphocyte responses to cells from normal autochthonous tissues. A slight degree of blastogenesis, which did not exceed an SI of 3.0, was observed. Consequently, we chose this value as a cutoff point to distinguish positive from negative blastogenic responses to tumor cells.

The relation of the blastogenic response to tumor cell dose is germain to the role of cell density in lymphocyte cultures in general (*15*). Maximum responses are achieved at a critical tumor cell-lymphocyte ratio; once this ratio is exceeded, responses decline. One can speculate that the decline in blastogenic response with increase in tumor cell dose is due to crowding and unfavorable culture conditions, with nutritional deprivation of the responding lymphocytes and, possibly, physical interference of excess number of tumor cells with the uptake of the isotope.

The association between the intensity of the blastogenic response to tumor cells and the stage of disease is a phenomenon of major clinical interest. Gross suggested that immunologic deficiency tends to parallel the extent of malignant disease (*18*). After tumor surgery, impaired immunologic reactivity, in the presence of little or no detectable disease, is associated with increased incidence of tumor recurrence and poor prognosis (*19*). Recovery of general immunocompetence and the associated "rebound and overshoot" in lymphocyte function have been recognized as favorable prognostic signs in patients with solid tumors and leukemia who receive chemotherapy (*20, 21*).

The present study suggests that in vitro, cell-mediated tumor immunity correlates quantitatively with the extent of disease; i.e., as the disease progresses, tumor immunity tends to decline. In contrast, the median response to PHA did not appear to be affected by the extent of disease in this group of patients. This dichotomy suggests several possibilities: a decrease in tumor antigenicity, a decrease in tumor-recognition capacity by the lymphocytes, or specific antigen or antibody-mediated suppression of tumor immunity in progressively growing tumors. Correlation between cell-mediated tumor immunity and stage of disease was also studied with cutaneous delayed-hypersensitivity reactions (*2, 22, 23*). Localized solid tumors or remission in leukemia was associated with positive delayed-hypersensitivity reactions to tumor

cell extracts. Also, the degree of blastogenic response of lymphocytes to autologous leukemic blast cells was correlated with the clinical status of leukemia patients (*24*). A higher SI was associated with an increased incidence of remission and a decreased incidence of relapse. In contrast, Hellström et al. found no difference in antitumor immunity (microcytotoxicity assay) between patients who became symptom free after a curative manipulation and patients who had progressively growing tumors (*25*). However, the presence of blocking serum factors did correlate with advanced disease. Also, their test system used cultured tumor cell lines, in which antigens may be grossly altered (*26*).

The complexity of serum effects deserves a critical comment. Our results differ from those of other investigators who found only blocking serum effects in patients with progressively growing tumors. In contrast to those investigators who used in vitro assays for serum effects (*27, 28*), we use fresh rather than cultured tumor cells, and we incubate both tumor cells and lymphocytes together, in the presence of serum for 7 days, rather than tumor cells alone for a few hours. Our system may be closer to the in vivo conditions. Our results suggest that serum from cancer patients can exert both inhibitory and facilitory effects on lymphocyte blastogenic response to tumor cells. The mechanisms by which serum factors may exert their effects are poorly understood. Elution studies in animal tumor models revealed that tumor cells may be coated by antibodies (*29*) or by antigen-antibody complexes (*30*) which correlated with the presence of inhibitory factors in the serum from the tumor-bearing host. Also, such eluates could suppress antitumor immunity in vivo (*31*). Facilitory serum factors were detected in patients who became symptom free after a curative manipulation (*25*). These "unblocking" sera abrogated in vitro the inhibitory effect of serum from patients with progressively growing tumors. In the rat polyoma system, serum "unblocking" effect in vitro may correlate with its antitumor activity in vivo (*32*).

In the current study, no correlation was observed between the presence of any serum factors and the extent of disease; in most cases, serum factors were nonspecific, as reflected by the simultaneous effects on responses to PHA and allogeneic leukocytes (MLC).

The demonstration that tumor cells can induce blastogenic response in vitro among autologous lymphocytes supports the concept that tumor cells do contain nonself antigens on their surfaces.

It seems likely that these antigens are weak, since they induce mild blastogenic responses compared to other antigens and mitogens. However, one cannot rule out the possibility that various factors may interfere with the process of recognition and that this leads to a failure to evoke a more intense response.

Weak responses may have also been the result of a failure to select the ideal tumor cell preparation for the stimulation of blastogenesis. Our results emphasize the superiority of viable compared to nonviable tumor cells and the superiority of irradiated compared to mitomycin C-treated cells. The superiority of viable tumor cells deserves further comment. In some experimental systems, viable tumor cells are more effective for immunotherapy (*33*). In our system, viable tumor cells may be superior because they furnish a better source of tumor-associated antigen than nonviable cells. Cells in the process of disintegration may have lost a great deal of the surface antigen and certainly will not be able to synthesize new antigen. Further treatment of such fragile cells with irradiation or mitomycin C may remove more antigen or further reduce their ability to synthesize a new one. Conversely, good cell viability and continued cell-surface antigen production may be required for vigorous stimulator cell activity, both in the MLC and the tumor cell stimulation of blastogenesis.

How mitomycin C reduces the blastogenic activity of both viable and nonviable tumor cells is not known. The only well-characterized activity of mitomycin C is to induce structural change in DNA and completely inhibit its synthesis (*34*). In one-way mixed lymphocyte reaction, mitomycin C-treated lymphocytes appear to be satisfactory as stimulator cells (*8*). However, in an animal system where F_1 hybrid lymphocytes are unreactive to parental strains of cells and yet are stimulatory in the MLC, their treatment with mitomycin C markedly reduced stimulator cell reactivity (*35*). In tumor

cells, it might be postulated that some changes in membrane properties caused by this drug may lead to the diminished antigenicity. Leakage of mitomycin C from treated tumor cells with subsequent suppressing effect on the responding lymphocytes is also possible, despite the extensive washing to which they were subjected.

Irradiation may also reduce cell-surface antigens, especially the weak ones, and thus reduce MLC (*36*). Studies of the one-way mixed lymphocyte reaction demonstrated satisfactory stimulation by lymphocytes irradiated with 4000 R. However, doses of $\geq$6000 R always diminished stimulatory activity (*37*). Ultraviolet irradiation of lymphocytes was also associated with their inability to stimulate allogeneic lymphocytes (*38*). A comparative study of irradiation and mitomycin C pretreatment of stimulator cells for the one-way mixed lymphocyte reaction resulted in 2 conflicting reports. One claimed the superiority for irradiated stimulator cells (*39*) and the other found no significant difference (*40*). In our system with tumor cells, irradiated tumor cells were superior to mitomycin C-treated tumor cells.

The question of whether a positive blastogenic response to tumor cells merely represents a primary tumor recognition in vitro or evidence of cellular immunity and memory cells in the peripheral blood is not answered. The current study furnishes a preliminary evidence to support the latter hypothesis. KCl-solubilized tumor antigen can stimulate autologous lymphocytes but does not stimulate normal subjects' lymphocytes. A similar phenomenon was observed in patients with leukemia (*41*). It is proposed that soluble antigen, in contrast to its cell-associated counterpart, can only stimulate blastogenesis among presensitized lymphocytes. Hence autologous lymphocytes responded in vitro to the soluble antigen because they were presensitized to the tumor in vivo, whereas allogeneic lymphocytes failed to respond in vitro because they were not presensitized to either tumor-associated or HL-A antigens which were probably also present in the solubilized extract (*42*). This phenomenon, in our opinion, strongly suggests that the blastogenic response of autologous lymphocytes to tumor cells is indeed a manifestation of cell-mediated immunity.

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PART III

LYMPHOCYTE STIMULATION

DISCUSSION

OUTLINE OF DISCUSSION—LYMPHOCYTE STIMULATION

I. Source of antigen
   A. Whole cells
      1. Preparation of cells—mechanical, enzymes, purification
      2. Preservation of cells—fresh versus frozen cells
      3. Control cells used
      4. HL-A antigens on solid tumor cells
   B. Antigenic extracts
      1. Different extraction methods—results with membranes and with soluble fractions
      2. Stimulation by HL-A antigens

II. Lymphocytes
   A. Preparation from whole blood
   B. Results with purification: Are granulocytes and/or monocytes needed?

III. Assay method
   A. Media, type of serum
   B. Controls used: What is background?
   C. Controls for whether test is technically satisfactory
   D. Formula for calculation; definition of positive reaction
   E. Reproducibility of assay from day to day
   F. Macro- versus micro-method
   G. Relationship of reactivity to clinical state, to therapy
   H. B- or T-cells

IV. Serum blocking
   A. Method used
   B. Effects of different concentrations of sera; effects of normal human sera versus fetal bovine serum
   C. Correlation of blocking with clinical state

R. Herberman: There certainly have been some differences in antigen used by various workers. What about the presence of granulocytes in the stimulating population after preservation? Do the polys survive and are they needed for stimulation?

R. Halterman: With regard to the freezing and thawing of granulocytes, these cells do not survive the process well and will destroy other cells in the media. This probably results from enzymatic digestion. This brings up a point about Dr. Santos' normal mixed-leukocyte culture (MLC) system. Do you concentrate your lymphocytes used as stimulating cells?

G. Santos: Yes.

Halterman: Because you concentrate your blast cells before placing them in culture, you're using a large antigen dose when you're looking for a response to the autologous tumor cells. You have to compare that response to concentrated normal lymphocytes when you are looking for either tissue- or tumor-specific antigens as a control.

J. Gutterman: We don't have many polys in most of our acute leukemia preparations. Usually, at the speed that we run the centrifuge, we get $<5\%$ and most are lost by freezing.

B. Leventhal: You ask whether these cells are necessary. Of course one would always like to have a standard responder and a standard stimulator in any of these tests to compare with the patient, ideally a frozen normal standard. Because of Dr. Mardiney's report about 1½ years ago in the *Journal of Experimental Medicine*, we did not try to react frozen cells against frozen cells in MLC. He reported that either the stimulating or the responding cell population had to be fresh, the presumption being that there was a required phagocytic cell that was perhaps lysed during the freezing. Does anyone have any more data on that subject? We've occasionally tried running frozen cells against frozen cells and have obtained variable results.

A. Hollinshead: Perhaps the macrophages are not as important as the macrophage factor, which might or might not be preserved depending on the type of freezing.

Santos: I don't have any more data, but if I understand you, frozen cells are better stimulators and we don't find that they stimulate any more or less. I also agree with the granulocyte viability. The cells, of course, are washed after they come out of the freezer. At times, we get as high as 5%, but generally we get 1 or 2% granulocytes by our methods.

Leventhal: No, they are good stimulators, but have you tried mixing and have you tried them as responders against frozen cells?

Santos: No.

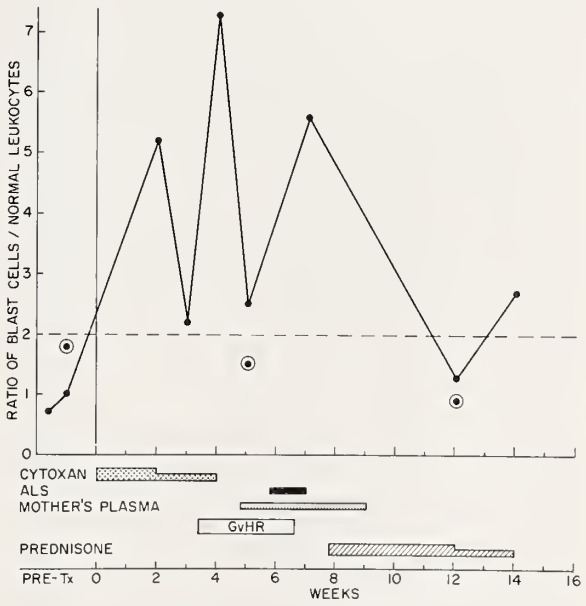
Herberman: Are granulocytes and/or monocytes needed in the responding population? Have people tried hard to remove all the granulocytes and monocytes from the responding population? How does that affect the stimulation in the tumor system?

Gutterman: We have not tried to separate the phagocytic cells from the responder population in our tumor studies. We sediment the blood with dextran and take off the layer rich in white blood cells. However, as most of you are aware, Dr. Hersh, in studies with Dr. Oppenheim and Dr. Leventhal [1] and in studies with Dr. Harris,[2] has shown that response to antigen is poor when one removes the macrophages. I would suspect the response to leukemia cells, and soluble leukemia antigen, would be poor without monocytes, but we haven't done this experiment.

Santos: I want to make sure that something is not said here that runs against someone's findings. First of all, I think our population is considerably older than Dr. Halterman's population and at least 80 or 90% of them are patients with acute myelogenous leukemia (AML). That's the only study I can refer to that may suggest we have something different. Our other data, with the HL-A identical, are what you expect, and fortunately we have found what you would expect. We really haven't looked at blocking antibody that critically. So, I don't think there's anything here that really runs counter to what has been said. This business of the blast cell stimulating more is intriguing and there may be a lot of reasons it might. But Dr. Herberman has apparently noticed this and several other people have, in the mouse system. For example, if we want to set up a positive chromium (^{51}Cr)-release assay, we inject DBA spleen cells into C57BL mice and look for ^{51}Cr release. Giving 10^7 cells intraperitoneally and looking a week later, we cannot really pick up ^{51}Cr release. On the other hand, if we give 10^6 L1210 cells and then take the lymphocytes of those C57BL mice, they will release ^{51}Cr not only from L1210, but also very nicely from the DBA spleen lymphocytes. The L1210 cell is a better immunogen for the normal H-2 antigen than are normal lymphocytes. I don't know if anyone else has looked at this.

Herberman: Most people's experience with cytotoxicity has been the same as yours, in that tumor cells are much better immunogens than normal cells. I don't know of anyone who can completely explain this finding; it may be related to the increased antigenic stimulus on the proliferating tumor cells.

Halterman: If I understand Dr. Santos' concern, i.e., finding a normal (HL-A identical) sibling reacting against the blast cells, it may be explained by the following (text-fig. 1). This text-figure gives results on an AML patient who received transplants of bone marrow from an HL-A-identical sibling. The response of the patient's lymphocytes toward his own blast cells is indicated. Before the transplant, the response was below significance; but after the graft, he developed a positive response, except during the time he was placed on prednisone in a period of graft-versus-host reaction. During the same time, the level of the response of the normal donor remained negative. This suggests that prior sensitization to the tumor-related antigen is definitely required. This patient is a 100% chimera by several markers, including immunoglobulins. We conclude that these are donor lymphocytes responding against the tumor cells. I think what you are saying is that your older age group may have had a greater opportunity to become exposed to this antigen in nature and therefore respond.



TEXT-FIGURE 1.—Seventeen-year-old male with AML, transplanted with bone marrow from his HL-A-matched sibling. Reactivity before the graft to his blast cells was negative. After the transfusion, he became positive, except for a short time when he was on steroids. The donor was always negative.

Santos: We had an HL-A-identical sibling who failed to release ^{51}Cr from the labeled tumor cells (acute monocytic leukemia cells). After the transplant, her cells having been in this environment did cause ^{51}Cr release from the tumor.

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Herberman: To return to the point that Dr. Leventhal brought up about frozen cells, one thing that Dr. Mardiney suggested in his article was that there is a differential loss of activity of the frozen cells in MLC versus response to soluble antigens by presensitized individuals. Perhaps some of the cells are needed for stimulation in a primary recognition system, whereas they are not needed in a presensitized situation. Does anyone have any information as to whether this distinction applies to tumor antigens? This distinction might be useful in terms of whether the response to tumor antigens reflects presensitization versus a primary recognition phenomenon. It might give more strength to the distinction that Dr. Gutterman suggested with the KCl extracts.

Gutterman: We found that patients who had poor response to their own leukemic cells frequently had poor response to streptolysin O (SLO). Yet their response to phytohemagglutinin (PHA) was excellent. Macrophages are necessary for optimal in vitro response to SLO but may not be necessary for optimal response to PHA. Therefore leukemia patients may sometimes have impaired macrophage function rather than lymphocyte function to account for one poor response to their leukemia cells. On the other side of the coin, patients may not respond in vitro to their own tumor cells, because the tumor cells themselves do not evoke a good response (i.e., are poorly antigenic). This is particularly true for patients with acute lymphocytic leukemia (ALL). In fact, ALL blasts sometimes do not stimulate allogeneic lymphocytes in the MLC.

S. Golub: A couple of things have indicated that these types of responses represent presensitized responder cells. One is the magnitude of some of them. Also, there is a report by Powles et al.[3] that, in leukemia patients who receive immunotherapy with X-irradiated cells, the response index went steadily up. This suggests that, as you increase the number of presensitized cells, you get a greater response.

Leventhal: Powles et al. reported in the *British Medical Journal* an increase in MLC response against autologous blasts as a response to immunization with those blasts. I have not talked with him about this directly, but I wonder whether the increase he observed in MLC response, rather than reflecting specific sensitization, might reflect the nonspecific "overshoot" in in vitro response that occurs 10–20 days after therapy has been discontinued. His patients were immunized as soon as therapy was discontinued.

G. Mavligit: Jehn et al.,[4] using a naturally solubilized antigen from a cystic melanoma, showed stimulation of autologous lymphocytes and also of lymphocytes from other patients with melanoma. They were unable to stimulate lymphocytes from normal subjects. This agrees with our findings using KCl-extracted tumor antigen in which the response of autologous lymphocytes to the antigen indicated the presence of a presensitized population of lymphocytes. In contrast, normal donor's lymphocytes did not respond to the antigen because they were probably not presensitized.

M. Sigel: Has anyone tried to inhibit the response of normal lymphocytes to cell-bound HL-A antigens by using soluble HL-A antigen? Although the solubilized HL-A weakly stimulates, it may still be capable of binding to the recognition sites on the lymphocytes and thereby prevent subsequent reaction of this type HL-A antigen on the cell surface. Have you done this experiment?

Gutterman: No.

Santos: Another possibility, just for speculation is that the response to HL-A indeed is a primary one and requires the membrane. It may have to be presented in a special way, and it may be that the KCl extract and other soluble antigens require presensitization. I don't know if that can be checked, but it would be a highly possible.

Mavligit: The functional difference between cell-associated HL-A antigen and the same antigen in its solubilized form is intriguing, and further studies are badly needed. Since red blood cells are known to be nonstimulatory to allogeneic lymphocytes, coating them with solubilized HL-A antigen may render them stimulatory.[5] Such an approach may provide invaluable information related to antigenic expression.

Santos: This can be checked right here at the National Cancer Institute because investigators could look to confirm that the KCl extract from their tumor patients does work. Secondly, they have been HL-A-typing people for platelet transfusions and should be able to test soluble HL-A antigens in the same way.

Gutterman: Dinitrochlorobenzene does not stimulate sensitized lymphocytes in 1-way MLC. But when attached to a cell, it does stimulate.

Herberman: This is a much different thing. You are talking about a hapten rather than a protein.

Leventhal: Along these same lines, Dr. Mavligit, do you see stimulation as you set up your normals against the normal tissues?

Mavligit: They were not normal subjects' but cancer patients' lymphocytes set up against their own normal tissues. Stimulation was observed, but always below an index of 3.0.

Leventhal: But you did not have unrelated normal individuals against normal tissues from cancer patients?

Mavligit: This was not done. I believe very strongly that we have to do more of these experiments using normal tissues to stimulate cancer patients' lymphocytes. We have not used any liver tissue, but I am very

[3] Powles RL, Balchin LA, Fairley GH, et al: Recognition of leukaemia cells as foreign before and after autoimmunization. Br Med J 1:486–489, 1971.

[4] Jehn UW, Nathanson L, Schwartz RS, et al: In vitro lymphocyte stimulation by a soluble antigen from malignant melanoma. N Engl J Med 283:329–333, 1970.

[5] Hardy DA, Ling NR: Effects of some cellular antigens on lymphocytes and the nature of the mixed lymphocyte reaction. Nature (Lond) 221:545–548, 1969.

suspicious about the liver because it may carry tissue-specific antigens.

R. Oldham: Dr. Mavligit, why didn't the frozen cells stimulate in the last experiment using the KCl extract?

Mavligit: These tumor cells were, unfortunately, not frozen in liquid nitrogen and, therefore, were not in optimal condition when thawed. The question of whether solubilized tumor antigen is superior or inferior to whole tumor cells in stimulating lymphocyte blastogenesis remains to be studied.

Herberman: Has anyone directly compared fresh and frozen cells? Is there any evidence for loss of antigenicity on freezing or decreased reactivity of the frozen cells?

Mavligit: I have emphasized from the beginning that we really insisted on studying fresh tumor cells, except for sequential studies in one patient whom we immunized with irradiated melanoma cells.

Halterman: We tried that situation and found that the frozen cells stimulate better, but that's on the basis of concentrating the antigen. You get rid of polys by freezing. If you concentrate the normal lymphocytes, then you are roughly talking about the same degree of stimulation.

Golub: Has anyone had any success using tissue culture cells in autologous systems, other than with lymphoblastoid cells?

Herberman: Is there any information with solid tumors, with the cells prepared by trypsinization or other types of enzymatic treatments? Does this adversely affect the stimulation of the lymphocytes?

Mavligit: There are several objections to the use of trypsin in the preparation of a single tumor cell suspension for immunologic studies. The most important one derives from a well-known observation among investigators of cell-surface components that treatment of any cells with the enzyme will change their agglutinability by concanavalin A, by a mechanism that involves alteration in cell-membrane properties.[6] Therefore we have only used mechanical means.

Herberman: In terms of the control cells, I think Dr. Mavligit brought up an important issue. It is important to see how the normal cells of the same morphologic type stimulate. If one indeed gets stimulation ratios of 2 or 3 with normal cells, and 3, 4, or 5 with tumor cells, can one say that he is looking at different phenomena, or are there just quantitative differences between the same antigens on the tumor cells and normal cells? Do you want to respond to that, Dr. Mavligit?

Mavligit: This is, of course, a critical point. At least 3 antigen systems have been detected in human tumors: 1) Embryonic antigens.[7,8] 2) Normal antigens which may be present in increased concentration in the malignant tissue.[9,10] This was also shown at this Conference by Dr. Koldovsky. 3) Tumor-specific antigens.[11] In one study with carcinoembryonic antigen (CEA), Gold's group could not demonstrate any significant stimulation of lymphocytes from patients with gastrointestinal cancer.[12] The possibility that increased concentration of normal tissue antigens in the malignant tissue may account for the stimulation of autologous lymphocytes cannot be ruled out completely. There is evidence that lymphoblasts from lymphoid cell lines derived from the peripheral blood of a normal donor could stimulate autologous lymphocytes.[13,14] Our own limited experience with KCl-extracted antigen suggests that we are dealing with tumor-associated antigen, since it stimulates only autologous, ostensibly presensitized lymphocytes but not normal donor's lymphocytes. The relationship between the normal tissue antigen present in malignant tissue and tumor-associated antigen is unknown. Quite possibly, tumor-associated antigen is merely an increased amount of a normal product resulting from any rapidly proliferating cells—malignant or benign.

Herberman: Have all these tests with the normal tissue antigens been done in cancer patients?

Mavligit: Yes.

Herberman: Let me suggest another possibility. There have been some suggestions, with cytotoxicity assays, that cancer patients are being immunized against tissue-specific antigens. If you reacted the lymphocytes of a patient with a benign disease against his biopsy tissue, would he be stimulated by the same type of tissue antigens?

Sigel: The extraction and storage of normal tissue antigens should provide a bank of control antigens to be used as standards for comparison of specificity of blastogenic reactions. Such controls are not yet possible for the cytotoxic reactions.

Leventhal: The problem of using a specific purified

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[6] Burger M: Cell surfaces in neoplastic transformation. Curr Top Cell Regulation 3:135–193, 1971.

[7] Gold P, Freedman SO: Demonstration of tumor-specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. J Exp Med 121:439–462, 1965.

[8] Klavins JV, Mesa-Tejada R, Weiss M: Human carcinoma antigens cross reacting with anti-embryonic antibodies. Nature [New Biol] (Lond) 234:153–154, 1971.

[9] Loisillier F, Buffe D, Tan KB, et al: Etude immunologique des épithéliomas mammaires humains. Ann Inst Pasteur (Paris) 109:1–21, 1965.

[10] Taylor G, Odili JL: Tumour specific T-like antigen of human breast carcinoma. Br J Cancer 24:447–453, 1970.

[11] Gentile JM, Flickinger JT: Isolation of a tumor-specific antigen from adenocarcinoma of the breast. Surg Gynecol Obstet 135:69–73, 1972.

[12] Lejtenyi MC, Freedman SO, Gold P: Response of lymphocytes from patients with gastrointestinal cancer to the carcinoembryonic antigen of the human digestive system. Cancer 28:115–120, 1971.

[13] Green S: Mixed leukocyte stimulation of normal peripheral leukocytes by autologous lymphoblastoid cells. Science 170:989–990, 1970.

[14] Birnbaun G, Siskind GW, Weksler ME: Autologous and allogeneic stimulation of peripheral human leukocytes. Cell Immunol 3:44–52, 1972.

tissue antigen as a control has been illustrated in the studies by both Dr. Mavligit and Dr. Gutterman and by our studies of HL-A. We all find that, if the patient is not sensitized, we get a very low level of response. I agree that, until we alter the purified antigens so that we can obtain a more consistent response, we have no positive control and no way of checking that our experiment is technically satisfactory, e.g., that something did not happen to our antigen while freezing to denature it.

Herberman: Does anyone have any information about attempts at stimulation with antigen extracts other than KCl? Is there something special about KCl or would other types of extracts work as well?

G. Klein: Two points about the source of antigens: In the studies performed by Stjernswärd and Vánky, as in your experiments, frozen tumor biopsies did stimulate if they were protected with dimethyl sulfoxide (DMSO) and stored in liquid nitrogen. Normal tissue biopsies used as controls, like skin in the sarcoma experiments or kidney in the kidney carcinoma experiments, did show occasional stimulation of the autologous lymphocytes up to levels exceeding the background (lymphocyte vs. lymphocyte) by a factor of 2–2.5. The corresponding lymphocyte-autologous, mitomycin C-treated, tumor-biopsy figures were between 4 and 5. I was most interested to hear that one could get a much better stimulation by the tumor and a greater differential versus the normal tissue if the biopsies were not treated with mitomycin C. Apparently, it is not really necessary to treat biopsy specimens, not adapted to tissue culture, with DNA inhibitors because they will not make much DNA anyway. As far as tissue culture lines are concerned, Stjernswärd and Vánky had a very curious difficulty, and I wonder if others have experienced something similar. Using parallel sarcoma and normal fibroblast-derived lines from the same patients, they found it very difficult to inhibit, by mitomycin C and/or by X-ray, DNA synthesis in both kinds of established lines. Is it true that continuously growing lines are less easily inhibited than freshly explanted lymphocytes for which the MLC test was originally worked out? The autologous tumor-cell membrane extracts that Dr. Herberman kindly sent us did not stimulate the lymphocytes of patients who were stimulated by the corresponding autologous, mitomycin C-treated biopsies at the 3–5 level. I was wondering how these extracts were prepared, also in relation to the KCl preparations.

Herberman: The extracts that we sent to Dr. Vánky were membrane extracts rather than soluble extracts. These did give positive skin tests in Dr. Stjernswärd's patients with renal carcinoma. Yet in the same patients they did not stimulate the lymphocytes. I think Dr. Leventhal had had the same experience, that these membrane extracts for some reason do not generally stimulate lymphocytes, but they can elicit delayed-hypersensitivity skin tests. I might also add, in terms of negative kinds of results, that Dr. J. Oppenheim here at the National Institutes of Health attempted to stimulate the lymphocytes of melanoma patients with membrane extracts and with conicated soluble extracts which Dr. Hollinshead had prepared. These materials were uniformly non-stimulatory. So, although KCl extraction was not tried, there might be some distinction between KCl and one of the other methods for solubilization.

Gutterman: Did you do simultaneous stimulation with the whole cells? This is very important in evaluating soluble antigen data. One should try to do stimulation with whole cells, because if you don't get response to soluble antigens one can still demonstrate blastogenic response to the viable cells.

Herberman: But your group obtained some results in the opposite direction, when you got stimulation with the soluble extract and not with the cells.

Mavligit: This experiment was done with frozen cells which may have lost their antigenicity.

Gutterman: In leukemia there generally was pretty good correlation.

Oldham: In response to the problem raised by Dr. Klein, tissue culture cells could be used in an MLC. The problem that they cannot be inhibited adequately with either X-ray or mitomycin C might be surmounted by using a combination of something like iododeoxyuridine and fluorodeoxyuridine which will inhibit tissue culture cells very effectively at the right concentrations. It can stop 90–100% of cell proliferation, and the cells will still remain viable for several days, probably long enough to do an MLTC.

Golub: I find Dr. Mavligit's experience with the untreated cells very interesting, and I'd like to pursue that a little further. Were you possibly looking at a kind of feeder phenomenon in which the addition of the lymphocytes increased DNA synthesis among the untreated tumor cells? Will the addition of mitomycin C-treated lymphocytes increase thymidine incorporation by the tumor cells?

Mavligit: The evidence against such feeder phenomena derives from our experiments using viable tumor cells separated on Ficoll-Hypaque density solution. Thymidine incorporation in lymphocyte cultures was higher with irradiated viable tumor cells than with untreated viable tumor cells. It is unlikely that adding lymphocytes to irradiated tumor cells would increase incorporation of thymidine.

Herberman: Which media have been used for the stimulation assays?

Mavligit: Minimal essential medium (MEM).

Herberman: Is everyone else doing it in MEM? Or are there some differences in the data with different types of media?

Gutterman: Drs. F. and M. Bach have data suggesting that medium 199 was superior to MEM in mixed lymphocyte cultures, but we've used primarily MEM.[15]

Golub: We compared medium 199 with RPMI 1640 and with MEM in our stimulation system with the Burkitt-cultured lines. RPMI supported the best response.

[15] BACH F: Session I—Methods for the study of mediators. *In* In Vitro Methods in Cell-Mediated Immunity (Bloom B, Glade P, eds.). New York, Academic Press Inc., 1970, 74 pp.

Herberman: What about serum? Is everyone using pooled human serum and does the serum concentration make a difference?

Mavligit: We used heat-inactivated sera. Patients' lymphocytes were cultured in autologous and normal donors' sera, and the donors' lymphocytes were cultured in autologous and in patients' sera. Facilitory and inhibitory serum effects were detected, but their interpretation is difficult without data with a reference serum like AB+. Both inhibitory and facilitory serum effects were mostly nonspecific, in that they were often detected collaterally in the mixed leukocyte reaction between the patient and his paired normal donor and also in the response to PHA.

Herberman: What about the specificity in leukemia patients, Dr. Gutterman? If you would use a serum that blocked the autologous leukemia system, would it block mixtures of leukemia cells with the lymphocytes of other patients?

Gutterman: As I mentioned, 3 of the 9 patients who had inhibition to their leukemia cells also had inhibition to PHA. However, there was no inhibition to the response to SLO. We have evaluated the allogeneic normal donor simultaneously in those studies, and in 3 of the 9 instances, the inhibitor serum blocked allogeneic lymphocyte response to the leukemia cells. So it was not always specific.

Herberman: If you tested several different individual normal sera, or sera from patients with other diseases, would there be a broad range of stimulation? If there were, then it would be difficult to make a statement about inhibitory effects or facilitory effects with the patients' sera if you're just working within a broad range.

Leventhal: I'm quite sure that, if you took a series of normals, you would get a slide that looks exactly like the one that Dr. McKhann showed. There are definitely normals in whose sera everyone responds worse. I think you'd have to use a pool and also have standard reference sera.

Herberman: Do you know if that's related to AB blood group or another factor like that?

Leventhal: I don't know.

P. Periman: I'd like to comment about the media. There are at least 2 other media that others should investigate. One would be the NCTC 135, a medium designed to grow cells without serum, and the other is Ham's F12, a medium designed to grow single cells. Enriched, highly defined media might make a difference in terms of the ability of tumor cells to divide.

Mavligit: Going back to the serum problem, there is a major methodologic difference between the lymphocyte blastogenesis assay and the microcytotoxicity assay. In the former, both lymphocytes and tumor cells are cocultivated with the serum in question for 7 days, while in the latter, tumor cells alone are incubated with the serum and only for 45–60 minutes. Therefore, any serum effects observed in the 2 systems are incomparable.

Leventhal: Just one point that Dr. Stewart presented at the AACR meeting in Boston. He found that he could get nice mitogen responses in his serum-free system, but he could not get a mixed lymphocyte culture response unless some serum was present in the system. He has done a lot of work in this area, and I don't know if it is profitable to explore the idea of a serum-free control system.

Sigel: Do any of these facilitory or inhibitory sera in the autologous situations contain soluble antigens? Dr. Viza and others have shown that there is leukemia antigen in leukemic sera. What effect do these sera have on normal lymphocytes?

Gutterman: That is a good question. Sometimes lymphocytes do have higher background counts. When this does occur, in autologous serum compared to allogeneic serum, certainly soluble antigen could account for some in vitro stimulation. This facilitation effect is difficult to interpret. For example, Al-Sarraf et al.[16] reported that, if one looks at PHA response in autologous serum compared to allogeneic serum, often there is a decreased stimulation of PHA in the latter compared to the former. So instead of seeing facilitation in autologous serum (say by soluble antigen), we may be seeing some type of inhibition by allogeneic serum. Further interpretation, I think, is rather difficult. To call it a facilitory serum, perhaps, is going just a little too far.

Herberman: How about the calculation of the data? It appears from the formulas shown that there are some differences in the way that Dr. Halterman and Dr. Leventhal are calculating their results, as compared to the group from M. D. Anderson Hospital and Tumor Institute.

Mavligit: Studies of lymphocyte blastogenic response to irradiated autologous leukocytes showed no difference from unstimulated controls. In fact, some inhibition was observed. Thus, we have decided to use counts per minute (cpm) of unstimulated control lymphocytes as the denominator in calculating the stimulation index (SI).

Halterman: Dr. Mavligit and I have talked about this point before. I do not believe that the controls used in a "2-way" system are adequate. There are 2 major considerations. 1) Cell density is not constant, and 2) there may be some blastogenic response from the stimulating cell population. I think the 1-way system is superior.

Gutterman: First of all, we always use unirradiated as well as irradiated leukemic cells as stimulator cells in our cultures, so we have both the 1-way and the 2-way systems. It is unlikely that the leukemic cells could be stimulated by the patient's own lymphocytes. They are not stimulated as responder cells in the mixed lymphocyte cultures, with PHA or SLO. We get definite decreased stimulation when the leukemic cells are irradiated even when we go down to 1000 rads. Kasakura

[16] Al-Sarraf MM, Sardesai S, Vaitkevicius VK: Effect of syngeneic and allogeneic plasma on lymphocytes from cancer patients, patients with non-neoplastic diseases, and normal subjects. Cancer 27:1426–1432, 1971.

and Lowenstein[17] showed that irradiated lymphocytes that had been frozen stimulate less well than irradiated fresh lymphocytes. So, freezing a cell may make it susceptible to membrane changes after irradiation. This may be important in how we prepare cells for active immunotherapy. Irradiation of cells from some animal tumors may decrease their immunogenicity.[18]

G. Klein: It's very important to distinguish between the nonspecific and the specific blocking effects. If specific, disease-related blocking effects can provide an important set of reference reagents that can be used to characterize specific groups of tumor-associated antigens. Vánky and Stjernswärd found they could block the reaction of autologous lymphocytes against solid tumor biopsies in the mixed lymphocyte–tumor interaction type of experiment. They found significant blocking with autologous serum in 31 of 38 stimulatory combinations in sarcoma patients, 3 of 3 in carcinoma patients, and 3 of 5 in brain tumor patients. Allogeneic sera from patients with "related tumors" blocked the reaction in 17 of 29 sarcoma and 8 of 11 carcinoma combinations. Unrelated allogeneic sera taken from tumor patients were inhibitory in 1 of 11 sarcoma and 2 of 6 carcinoma combinations. Healthy donor sera were inhibitory in 6 of 34 sarcoma, 0 of 7 carcinoma, and 1 of 4 brain tumor combinations. In summary, autologous patients' sera were inhibitory in 37 of 46 combinations (80%), allogeneic sera from patients with "related" tumors in 25 of 40 combinations (60%), sera from patients with "unrelated tumors" in 3 of 17 combinations (18%), and healthy donor sera in 7 of 45 combinations (13%). This indicates a certain degree of specificity, and it may be worth while to use the blocking reaction for the characterization of some tumor-associated antigen-antibody systems.

Leventhal: Relative to what? What was the standard serum?

G. Klein: Pooled normal AB serum.

Herberman: If antibodies are blocking, can one get titration curves with blocking sera?

G. Klein: It should be possible. Due to limitations in available lymphocyte and target cell numbers, this has not yet been done, but it should be.

H. Churchill: Dr. Klein, have you examined the specificity of the blocking effect? Is stimulation by other antigens or PHA influenced by these sera?

G. Klein: Yes. PHA and allogeneic combinations give a much higher degree of stimulation. Of course, a negative blocking test is not very meaningful.

Mavligit: Dr. Klein, what are your criteria for serum inhibition of lymphocyte blastogenesis?

G. Klein: As a positive SI, they take values ≥ 3, after the means of triplicate determinations are calculated. A positive blocking is half this value or less.

Herberman: The next issue is the statistics of lymphocyte stimulation. Why is a particular stimulation ratio chosen as the positive cutoff? Is statistical analysis done on each experiment to see whether this particular cutoff is valid?

Halterman: Probably our best piece of data deals with our twin experiments. We know these pairs are HL-A identical and never see stimulation greater that a ratio of 2. We have used this level in determining significant stimulation with tumor material; however, this figure may be too high. With greater numbers, one may be able to go below 2 and demonstrate significant stimulation.

Santos: I hope I understood the question. When we say 2, this is about where it comes out. We accept significance with the Student's t test at the 5% level, but it's very rare that we find a ratio <2 that is significant by these criteria.

Herberman: Are there some that are 2 and that are not significant?

Santos: In our experience, no.

Herberman: Are there some ratios that are 1.5 or 1.6 that are statistically significant? Is it fair to consider those as negative reactions?

Gutterman: We have counted ≥ 2 as positive, but I think that one should also report the exact SI, rather than just "positive" or "negative." We find that with 2, the significance is at the 95% level of confidence. Occasionally, when we use autologous remission lymphocytes as stimulator cells, we see stimulation. I have never seen an SI >3, though. Of course, one might have circulating blasts in that population of cells, and morphologically we cannot recognize them as such.

Herberman: You do see stimulation by autologous remission lymphocytes that would be significant?

Gutterman: That is a rather unusual occurrence, but it has happened in at least 2 instances. At times what are called remission lymphocytes may really be circulating leukemia cells. Alternatively, remission lymphocytes could carry a leukemia antigen on their surfaces. But this is unusual in our experience.

Golub: We all use the response index as a convenient way to report the data, but clearly an increase in counts from 100 to 300 is very different from an increase from 1,000 to 3,000 with the latter representing a lot more DNA synthesis. Have people gone back and looked over their data using response indices and seen if the pattern holds true in terms of raw cpm?

Leventhal: The reason we stick to ratios is that we ran a small number of unrelated individuals once a week for 4 or 5 weeks. And a couple of these individuals had a tremendously variable baseline, and the ratios of stimulation tended to be much more constant than the cpm.

Golub: But how about the difference in cpm? Does it remain the same?

Leventhal: When I said that the baseline varied

[17] Kasakura S, Lowenstein L: Irradiated and preserved leukocytes in mixed leukocyte cultures. Proc Soc Exp Biol Med 125:355–360, 1967.

[18] Wepsic HT, Kronman BS, Zbar B, et al: Immunotherapy of an intramuscular tumor in strain-2 guinea pigs: Prevention of tumor growth by intradermal immunization and by systemic transfer of tumor immunity. J Natl Cancer Inst 45:377–386, 1970.

tremendously, I meant that in one particular individual the baseline varied from 300 cpm on 1 day to 3500 on another, and the stimulated counts then—against a particular but the same unrelated individual—were 3000 on the first and 30,000 on the next day, which means that the ratio went up to 10. In most individuals, however, the baseline stays the same and the stimulated counts stay the same and you can use either cpm or ratios. We need more data on patients like this with baseline variance. In the same people run against one another on different days, but in a very small group and individuals, the ratio seemed to be a more constant number or a better reflection of constant genetic distance in the MLC.

Herberman: Is there information about this with the tumor patients? If you performed the test with the same patients for 3 days in succession, what would the variability be in the results, either in the raw counts or in the stimulation ratio?

Mavligit: We have not done it on a day-to-day basis, but we followed a few patients with sequential studies while they were receiving chemotherapy. No significant changes were noted in the SI. In most cases there was a good correlation between the net cpm and the SI. However, occasionally (e.g., Hodgkin's disease) the baseline cpm of unstimulated lymphocytes are high and so are the cpm after stimulation with tumor cells. In such cases, the SI is more meaningful than the actual cpm in reflecting the blastogenic response. For comparison between 2 separate studies, the index is superior to the actual cpm because it accounts for the variability in the baseline.

Gutterman: Dr. Golub's point is very interesting. Since one can alter the SI with a small change in the baseline lymphocyte count, it's very important when reporting the data that both the SI and the cpm be reported.

J. Holland: I am not convinced of the long-term integrity of cells under nitrogen freezing. We have 2 pieces of data on that: In a set of identical twins, one normal and the other with acute myelocytic leukemia, sequential mixed leukocyte cultures with frozen blasts indicated that the normal twin, in fact, did respond to the leukemic twin, but over time of blast storage this response appeared to deteriorate. Dr. G. Bekesi has studied the freezing of mouse leukemic cells which are subsequently treated with *Vibrio cholerae* neuraminidase. Their immunogenicity is assessed by challenging immunized mice with fresh leukemia cells. Cells that had been long-frozen in 10% DMSO in liquid nitrogen do not immunize well, whereas the fresh enzyme-treated cells can immunize against a challenge of a million leukemic cells. We are not convinced that the antigenicity of the cell membrane is left intact after months of storage.

Herberman: Would someone else like to comment on their experience with prolonged freezing in liquid nitrogen as to whether cells lose antigenicity after long storage?

Halterman: Using normal lymphocytes, we have not noticed a reduction in their ability to stimulate in MLC after 18 months of storage in liquid nitrogen.

Holland: Are those normal lymphocytes? Have you looked at leukemic cells?

Halterman: We have, but I can't give you the exact time. We have data over 18 months in which blast cells still stimulate unrelated individuals. I don't think we have had a patient in the early series survive that long, so we have not been able to look at the autologous situation, about which you are asking. This question is important, particularly when one uses these cells for immunotherapy. Do we still have a viable antigen?

Santos: Certainly, hematopoietic cells in the mouse and in man have been easily stored. They can be kept for long periods. We have kept them for 6 months. They really don't lose very much if one does a spleen-colony assay. We have the same type of differential on the cell suspensions. I know this does not say anything about their antigenicity. Cells that we have stimulated to work in the Jerne plaque technique we can keep up to 6 months with very little loss in their efficiency. We have stored lymphocyte panels for tissue typing and have gotten the cells to react with the same patterns several months later, but this is not answering whether they are good immunogens or not. I can't answer that.

Gutterman: We have not been impressed that cells stored for a year stimulated any differently from cells stored for short terms. I support the fact that this needs to be looked at very critically.

Leventhal: It has been our experience that, if someone faints while we are drawing the blood, or if a person who doesn't give blood very often has blood drawn several days in a row, their reactivity falls off. I think they worry and release a lot of steroids. With laboratory personnel or patients who are used to having blood drawn, you can draw blood day after day and get a consistent response, but if you get normal volunteers and try to draw blood from them Monday through Friday, by Friday you might as well not bother. This is a small point, but we have observed it relatively consistently; if we're going to try to get normal standards, we should not try to do it in that fashion.

Halterman: What does the height of stimulation in the MLC really mean? Leukocytes stored in liquid nitrogen will stimulate very nicely and can be used as a control for any one individual. Hopefully, this control may solve one variable and keep HL-A distance constant. There are, however, at least 2 variables operating at best, using an MLC assay. Dr. Gutterman, how do you interpret the height of a response and do you arbitrarily pick 12 days? Possibly at day 20, you may be seeing a higher response as in the slides I have shown. It may depend more on the relationship to chemotherapy than the actual individual's response per se.

Gutterman: In our serial studies we are as consistent as possible, so that around 10 days after the completion of the last course of chemotherapy we do our mixed lymphocyte cultures. Our serial studies are fairly consistent from month to month in our patients. That is,

patients with high SI's in remission studied over several months almost always maintain high SI's. Those with a negative blastogenic response almost without exception maintain a negative response when studied over several months. Of great interest in that we have had 2 patients with positive and high SI's over several months who eventually converted to negative. Both these patients had relapses within 6 weeks of their negative study. So we are starting to see some very good correlation between the blastogenic response to the leukemia cells in remission and their subsequent clinical course.

Halterman: And they're all on the same type of chemotherapy?

Gutterman: These patients are on basically the same type of intermittent chemotherapy, but there are some slight differences. They are on cytosine arabinoside, prednisone, and vincristine. Some patients are also on daunorubicin and/or are on Cytoxan. All the studies I reported were done on patients on intermittent chemotherapy. Because there are different immunosuppressant effects of the chemotherapy, the persistence of immunosuppression can vary with each type of chemotherapy. Generally, our studies have shown that the immunosuppression has been recovered by day 10 after a 5-day course of chemotherapy with cytosine arabinoside.

Periman: I have kept tissue culture cells for over 3 years, could unfreeze them, and grow them without any differences in their growth, cloning efficiency, or protein production. And also in the literature there is quite a difference between cell types with regard to how fast they ought to be frozen down. Some cells can be plunged directly into liquid nitrogen and survive; other cells have to be frozen at $-1°$/minute; and others, at faster or slower rates. So there may be some variation in that regard.

Gutterman: Once the cells are taken out of liquid nitrogen, should they be rapidly thawed and washed? We thaw and wash to remove the DMSO rapidly.

Periman: Most people's experience has been to thaw them very rapidly and to wash them rapidly to eliminate DMSO.

N. Levy: I would disagree with the rapid washing to eliminate DMSO. We do thaw rapidly but immediately dilute the cell suspension 1:20 in medium containing 20% fetal calf serum and immediately seed the cells in culture vessels. We have found recently thawed cells to be extremely labile to centrifugation and have lost >90% of the cells after but 1 wash at 150 × *g*.

Herberman: I don't think there is real disagreement. Adding tissue culture medium dilutes out the DMSO. We have also found that centrifuging the frozen cells can damage them.

Halterman: As you know, DMSO passively enters the cell but not as fast as water. When the cell suspension is diluted to remove DMSO, it is best to do so slowly to avoid osmotic lysis.

PART IV

SKIN TESTS

Delayed Cutaneous Hypersensitivity Reactions to Extracts of Human Tumors [1, 2]

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SUMMARY—Skin tests for delayed hypersensitivity have been used to study the cellular immune response of patients to tumor-associated antigens. The time course and histologic appearance of the reactions have been consistent with delayed-hypersensitivity reactions. The major emphasis in these studies has been to define the specificity of the reactions and to correlate test results with the clinical course of disease. Normal tissue extracts at high protein concentrations ($>$0.3 mg/0.1 ml) have produced positive reactions in patients and in normal volunteers. It was unclear whether these reactions were due to nonspecific irritation or to a low level of autoimmunity to tissue antigens. After this finding, extracts were tested at concentrations of $\leq$0.1 mg protein/0.1 ml. In Burkitt's tumor and in acute leukemia, these skin tests have detected antigens which appear to be tumor specific. Reactivity of patients has correlated well with the clinical state of the patients. In intestinal cancer, a carcinoembryonic antigen has been found, which is distinct from the carcinoembryonic antigen of Gold. In carcinoma of the breast, skin reactions have been elicited by extracts of normal tissues and by tumor preparations.—Natl Cancer Inst Monogr 37: 189–195, 1973.

IN THE PAST few years, evidence has been increasing for cell-mediated immunologic reactions against antigens associated with human neoplasms. Most studies have been performed with in vitro assays. It has also been possible to measure cellular immunity in vivo, by skin testing for delayed-hypersensitivity reactions. This in vivo test might be a closer reflection of the state of immunity in tumor patients than an in vitro assay. Hughes and Lytton (*1*) and Stewart (*2*) prepared crude extracts of carcinomas and skin-tested the autochthonous patients. Reactions were positive in about 25% of the patients. The reactivity of their patients did not correlate well with clinical state. In a later study, Stewart and Orizaga (*3*) found that breast cancer patients with positive reactions actually had decreased survival compared to the survival of negative skin reactors. However, the specificity of the observed reactions was not well characterized: Some control extracts produced positive skin reactions. In addition, bacterial contamination of some of the preparations could have contributed to the reactions (*2*).

Our group has also observed delayed skin reactions to extracts of a variety of human

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tumors. The major emphasis in these studies has been to define the specificity of the reactions and to correlate test results with the clinical course of disease. The problem of specificity has been approached in several ways: 1) tests of patients with comparable extracts of normal tissues from the same patient and from patients with nonmalignant disease; 2) tests of patients with a different type of tumor; and 3) tests of control tissue extracts in normal volunteers and in patients with nonmalignant disease.

MATERIALS AND METHODS

Subjects studied.—Patients with acute leukemia were studied at the Clinical Center of the National Institutes of Health (NIH). Patients with Burkitt's lymphoma were studied at the Uganda Cancer Institute, Kampala, Uganda. Patients with carcinomas of the breast or colon were studied at George Washington University Medical Center.

Skin tests were also performed on 25 normal adult volunteers, 25–46 years old, and on 12 siblings of pediatric patients, 3–12 years old.

Preparation of membrane extracts.—A sterile technique was used for all procedures. Leukemia cells and normal leukocytes were obtained from heparinized peripheral blood or bone marrow, after sedimentation of the erythrocytes by gravity or by Plasmagel (Laboratoire Roger Bellon, Neuilly, France). Solid tumors, obtained at biopsy, were minced, and the brei was pressed through a 60-mesh stainless-steel sieve. Membrane extracts were perpared by freeze-thawing and low-ionic strength extraction (table 1), a modification of the methods of Davies (*4*) and Mann et al. (*5*). Sterility of the final preparations was tested by culture on blood agar plates and in thioglycollate broth. Only sterile extracts were used for skin testing. Extracts prepared in this manner contained a high yield of cell-surface membranes, as measured by the ability to absorb anti-HL-A antibodies from a variety of leukocyte alloantisera.

TABLE 1.—*Preparation of membrane extracts*

1)	Preparation of single cell suspension in 0.14M NaCl.
2)	Freezing at −70° C and thawing.
3)	Extraction* with 0.14M NaCl.
4)	Extraction* with 0.07M NaCl.
5)	Extraction* with 0.035M NaCl.
6)	Obtaining membrane pellet from pooled supernatant fluids by centrifugation at 105,000 × *g* for 1 hour.
7)	Suspension of membranes in 0.14M NaCl.
8)	Adjustment of concentration with 0.14M NaCl, as determined by protein concentration (*6*).

*Extraction consisted of suspension in saline, incubation at 4° C for 10 minutes, and centrifugation at 500 × *g* for 10 minutes.

Preparation of skin-reactive soluble antigens.—Solubilization of the membrane extracts with low-frequency sonication, with subsequent fractionation of this material by Sephadex gel filtration, has been described (*7–9*). The procedures are summarized in table 2. Polyacrylamide gel electrophoresis was performed by a gradient technique, as previously described (*10*). All soluble fractions were passed through a Millipore filter to ensure sterility.

TABLE 2.—*Separation of skin-reactive soluble antigens from membrane extracts of tumors*

Membrane extract
↓ Low-frequency sonication for 2 intervals of 3 minutes each; sedimentation of residual membranes at 100,000 × *g* for 60 minutes.
Soluble material sonically vibrated
↓ Sephadex G200 gel filtration; skin tests of pooled and concentrated fractions.
Skin-reactive soluble antigen fraction
↓ Polyacrylamide gel electrophoresis.
Isolated protein fraction

Administration and interpretation of skin tests.—Skin tests were performed by intradermal inoculation of 0.1 ml, usually on the patient's back. At the same time as the tests with tissue extracts, the patients were also tested with a battery of standard recall antigens, to determine their general ability to manifest a delayed skin reaction. The following antigens were used: *Candida* (dermatophytin "O" 1:100, Hollister-Stier Laboratories, Spokane, Wash.); SKSD (streptokinase 40 U/streptodornase 10 U, Varidase, Lederle Laboratories, Pearl River, N.Y.); mumps (Eli Lilly & Co., Indianapolis, Ind.); and trichophyton (dermatophytin 1:30, Hollister-Stier Laboratories).

The delayed skin reactions were maximal at 24–48 hours. A positive reaction was defined as $\geq$5 mm of induration at 48 hours after inocula-

tion. Over 100 skin-punch biopsies of positive and negative reactions were carried out. Agreement was generally very good between clinical readings and biopsies as to typicality for delayed hypersensitivity; i.e., there were lymphocytes and histiocytes clustered around small blood vessels in the upper dermis.

RESULTS

Delayed Skin Reactions to Recall Antigens

We analyzed the reactivity of an initial series of 76 tumor patients at the NIH Clinical Center (mainly patients with acute or chronic leukemia) to the battery of standard recall antigens. Table 3 summarizes the responsiveness of these patients and also of 29 normal controls. Of the cancer patients, 21% were anergic. Among the nonanergic patients, the mode response was to 1 of the 5 antigens; 83.4% responded to only 1 or 2 antigens. In contrast, all of the controls reacted to at least 1 of the antigens, and half reacted to ≥ 3.

This battery of antigens has been useful in evaluating the general ability of the patients to have a delayed skin reaction. Patients unreactive to these antigens have not reacted to tumor extracts.

Reaction to Antochthonous Extracts—Effects of Protein Concentration

This initial group of patients was tested with various concentrations of membrane extracts of autochthonous tumor cells and, when possible, of normal (or remission) peripheral blood leukocytes (*11*). Of the 60 nonanergic patients, 42 (70%) gave positive reactions to at least 1 of the concentrations of tumor extract. However, some of these patients also reacted to the control extracts. When the normal controls were tested with extracts of their autochthonous leukocytes, 76% also gave typical delayed skin reactions. Most of the positive reactions to the extracts of normal leukocytes, in both the patients and controls, were elicited by preparations with high protein concentration. None of the patients and only 10% of the normals reacted at protein concentrations of 0.33 mg protein/0.1 ml or less. No normal subject reacted to extracts at 0.1 mg protein/0.1 ml.

In contrast to the decreased reactivity of the controls to extracts at low protein concentrations, 50% of the patients gave positive reactions to extracts at the same concentrations. This degree of responsiveness was highly significantly different from that of the normal controls ($P<0.0003$, χ^2 analysis). Because of the marked differential activity at low protein concentrations, all subsequent tests on tumor patients were performed at 0.1 mg protein/0.1 ml or less.

Skin Tests on Patients With Acute Leukemia

Membrane extracts of autologous leukemic blasts have produced positive skin reactions in patients with acute leukemia (*11, 12*). In patients with acute lymphocytic leukemia (ALL) in remission, reactions were positive in most, whereas only 1 reaction was positive in those with ALL in relapse (table 4). A similar pattern was found in patients with acute myelogenous leukemia (AML), though the incidence of reactivity in relapse was higher. No positive

TABLE 3.—*Responsiveness to skin testing with standard antigens*

	Number (% nonanergic patients responding)					
	1	2	3	4	5	0(anergic)
	Number (nonanergic patients responding)					Number (% total patients tested)
Patients						
<15 years old	7(70)	2(20)	1(10)	—	—	1(9)
>15 years old	21(42)	20(40)	6(12)	3(6)	—	15(23.1)
Total	28(46.7)	22(36.7)	7(11.7)	3(5.0)	—	16(21.1)
Normal volunteers						
<15 years old	2(50)	2(50)	—	—	—	0(0)
>15 years old	2(7.0)	8(32.0)	10(40.0)	4(16.0)	1(4.0)	0(0)
Total	4(13.8)	10(34.5)	10(34.5)	4(13.8)	1(3.4)	0(0)

reactions have been elicited by extracts of remission cells, when tested at the same protein concentration (0.1 mg protein/0.1 ml). In 15 of 18 patients in whom serial skin tests with tumor extracts were performed, skin test results correlated completely with clinical state: Tests were positive in remission and became negative in relapse. Text-figure 1 shows the representative results from 3 patients, in whom 4–6 tests were done. When the patients went back into remission, the skin tests became positive again. These changes in reactivity to the membrane extracts could not be accounted for by a generalized change in skin reactivity. Tests with the battery of recall antigens were not affected by the changes in clinical condition; the same tests were positive during remission and relapse.

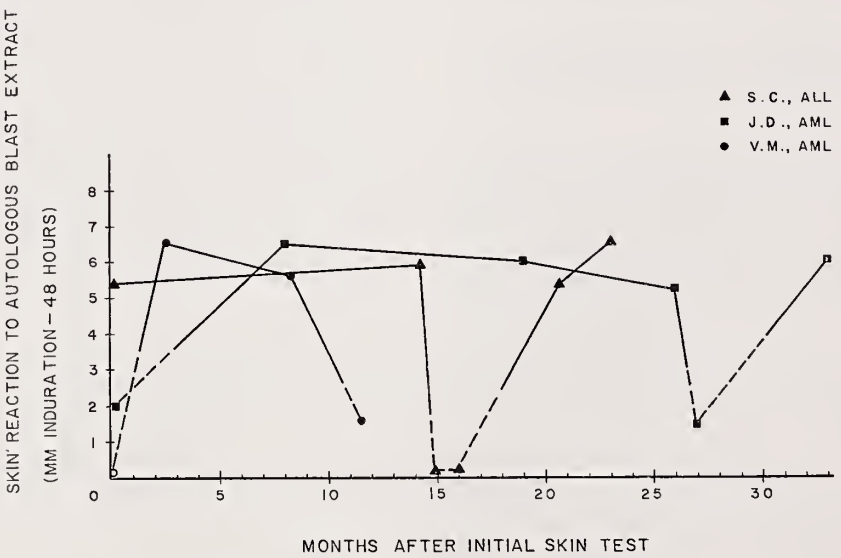
The results of these serial skin tests also provide some information about the lack of detectable immunization by the membrane extracts. Repeated skin tests at various times during remission did not produce increased skin reactions.

We have recently tested with membrane extracts of cells from allogeneic leukemia patients. Each recipient was inoculated with 3 or 4 preparations. Whenever possible, tests were done with extracts of the blast cells and remission cells from the same patient. The results are summarized in table 5. Reactions were positive in both ALL and AML patients, in response to extracts of blast cells from the same disease. However, the incidence of reactivity was lower than that seen with autochthonous extracts. In addition, most reactive patients were positive to only 1 of the test preparations. The antigens recognized in these tests appeared to be leukemia associated: Extracts from remission leukocytes, from blasts of the other type of leukemia, and from leukocytes of normal individ-

TABLE 4.—*Skin tests in patients with acute leukemia with membrane extracts of autochthonous cells*

Test material*	Tests positive/total No. of tests	
	Remission	Relapse
ALL		
Leukemia cells	18/23	1/16
Remission cells	0/14	0/5
AML		
Leukemia cells	18/20	7/19
Remission cells	0/5	0/4

*All tests were performed with 0.1 ml membrane extracts at protein concentration of 1 mg/ml.



TEXT-FIGURE 1.—Skin tests with membrane extracts of autochthonous leukemia cells, performed at various times after initial skin tests. *Solid lines* indicate periods of clinical remission. *Dashed lines* indicate periods of relapse.

uals gave negative reactions. Allogeneic extracts of normal leukocytes also did not elicit any positive reactions in 23 normal controls. This lack of reactivity to allogeneic normal cell extracts was somewhat surprising, since in many combinations, there were known HL-A antigeneic differences. The allogeneic extracts also did not appear to produce sensitization. Repeated tests with the same extracts did not produce increased skin reactions.

TABLE 5.—*Skin tests with membrane extracts of allogeneic cells*

Disease of recipient	Type of extract	Number of patients with positive tests/total No. of patients tested
ALL, remission	ALL blasts	19/31
ALL, remission	ALL remission leukocytes	0/10
ALL, remission	AML blasts	0/8
ALL, remission	Normal leukocytes	0/8
AML, remission	AML blasts	3/8
AML, remission	AML remission leukocytes	0/5
AML, remission	ALL blasts	0/6
AML, remission	Normal leukocytes	0/5
Normal	Normal leukocytes	0/23

Studies on Patients With Burkitt's Lymphoma

In collaboration with Dr. Leroy Fass, Dr. Avrum Bluming, and Dr. John Ziegler, we tested a series of patients with Burkitt's lymphoma with extracts of autologous tumor and of normal lymphocytes (*13, 14*). As with the acute leukemia patients, reactivity to tumor extracts was correlated with the clinical state of the patients. Before treatment, with tumor present, only 1 of 16 patients gave positive reactions. In remission, about half of the patients gave positive reactions (table 6). The incidence of relapse or mortality was the same in skin-test-positive and -negative patients. However, those patients with positive reactions remained in remission significantly longer than those with negative reactions. Seven patients with positive reactions subsequently had a relapse and then had negative reactions. Upon reinduction of remission, positive reactions were again seen.

TABLE 6.—*Skin tests in patients with Burkitt's tumor: Correlation with clinical course of disease*

	Skin test in remission	
	Positive	Negative
Total No. of patients	15	15
Patients with subsequent relapse	7	9
Mortality*	3	5
Remission duration†	25	6

*Number of patients.
†Median in weeks.

Studies on Patients With Intestinal Cancer

Skin testing has also detected tumor-associated antigens in patients with intestinal cancer (*8, 9*). It has been possible to solubilize the specific skin-reactive antigen from the tumor membranes by low-frequency sonication. Patients have reacted both to autochthonous and to allogeneic preparations. Table 7 shows the results of an allogeneic test with pooled carcinoma extract. No reactions were seen with comparable extracts from normal tissues. Extracts of fetal intestine and liver, obtained during the first and second trimesters, also gave positive reactions. The Sephadex fractions containing the skin-reactive antigen usually contained the carcinoembryonic antigen (CEA) of Gold, as detected by inhibition in the radioimmunoassay (*15*). However, by further fractionation of the antigens by acrylamide gel electrophoresis, CEA was clearly separated from the skin-reactive antigen (*9*). Purified CEA did not produce positive skin reactions. Therefore the skin-reactive antigen in intestinal cancer appears to be a carcinoembryonic antigen different from the CEA of Gold.

TABLE 7.—*Allogeneic skin tests with soluble fractions from pooled intestinal cancer and fetal liver extracts*

Preparation	Protein concentration (μg/0.1 ml)	Delayed skin reaction	CEA of Gold
Carcinoma of colon:			
Sephadex fraction I	35	0	0
Sephadex fraction II	20	+	+
Sephadex fraction III	25	±	0
Fetal liver:			
Sephadex fraction I	25	0	0
Sephadex fraction II	13.5	+	+
Sephadex fraction III	20	±	0

Skin Reactivity in Breast Cancer

Studies in breast cancer have pointed toward a tissue-associated, in addition to a tumor-associated, antigen (Alford, Hollinshead, and Herberman, unpublished observations). With membrane extracts (table 8), tumor-associated reactivity was observed. With only one exception, breast tumor membranes alone produced positive reactions. However, soluble fractions, obtained from the membranes by sonication and separation on Sephadex G200, produced a different pattern of reactions (table 9). Patients with breast cancer reacted to soluble fractions from breast tumor, normal breast from the tumor patients, and to normal breast tissue from patients with benign disease. Patients with benign breast disease also had positive reactions to the normal breast extracts, but patients with other benign diseases did not. Further studies of the soluble fractions by acrylamide gel electrophoresis are now in progress, in an attempt to separate the tissue-associated antigens from the tumor-associated antigens.

TABLE 8.—*Delayed-hypersensitivity skin reactions to breast membrane extracts*

Type of patient	Membrane extracts (Number of positive tests/No. of patients tested)		
	Cancer of breast	N-Ca breast*	N-N breast†
Breast cancer			
Autologous	3/9	0/4	
Allogeneic	3/4	0/4	1/5
Other cancer	0/4	0/3	0/2
Benign disease			
Breast			0/3
Other			0/2

*Normal breast tissue from mastectomy specimen of cancer patient.

†Normal breast tissue from patients with benign breast disease.

TABLE 9.—*Delayed-hypersensitivity reactions to soluble fractions from breast membranes*

Type of patient	Soluble fractions* (Number of positive tests/No. of patients tested)		
	Cancer of breast	N-Ca breast†	N-N breast‡
Breast cancer			
Autologous	5/6	3/5	
Allogeneic	4/4	3/4	4/5
Other cancer	2/4	1/4	1/4
Benign disease			
Breast			3/4
Other			0/3

*Membrane extracts were sonically vibrated, and soluble proteins were separated on Sephadex G200. Reactive fraction contained proteins in 42–56,000 molecular weight range.

†Normal breast tissue from mastectomy specimen of cancer patient.

‡Normal breast tissue from patients with benign breast disease.

DISCUSSION

Skin testing for delayed hypersensitivity has been a useful method for studying cellular immune responses to human tumor-associated antigens. In patients with acute leukemia and with Burkitt's tumor, skin testing has detected reactivity to antigens which appear to be tumor specific. With carcinoma of the breast, antigens not related to the tumors have also been demonstrated. The antigens associated with these diseases need to be separated further, to determine whether tumor-specific and tissue-specific antigens are present.

In addition to the skin tests with autochthonous tumor extracts, tests with allogeneic preparations have also given some positive reactions. It is often assumed in tumor immunology that reactions to common antigens are suggestive of a viral etiology for the tumor. Although this is one major explanation for reactivity to allogeneic antigens, the present work illustrates that at least 2 other causes exist. Carcinoembryonic antigens can be found in a variety of tumors, presumably due to derepression in the tumor of host genetic information. In intestinal cancer, such antigens can elicit cell-mediated immune responses. Tissue- or organ-specific antigens can also account for common antigenicity in tumors. This has been a rather neglected category, which may actually be the basis for many observed reactions.

Since skin testing is an alternative to the various in vitro assays for cellular immunity, it is worthwhile to consider the relative advantages and disadvantages of this method. The in vivo assay has the following advantages: 1) It is a relatively simple technique, which does not require specialized equipment or extensive training of personnel. 2) It allows use of tumor

tissues taken directly from the patient, obviating the need for tissue culture. This also makes it much easier to perform controls with appropriate normal tissues. In contrast, it has been difficult or impossible in most cases to have available, for in vitro testing, the tissue culture cells of the normal cells of the same morphologic type as the tumor. 3) The skin tests may be a closer reflection of the in vivo immune state. When skin tests and 2 in vitro assays were used to study patients with acute leukemia, only the skin tests correlated well with the clinical state of the patients (*16*).

The skin test assay has the following possible disadvantages: 1) Precise quantitation of reactivity is difficult. The tests are arbitrarily defined as positive when there is ≥ 5 mm of induration at 48 hours. Some reactions have a peak earlier or later than 48 hours. Tests with various concentrations of the same material do not give a clear dose-response curve. One of the problems associated with this is the inability to measure the depth of induration in addition to the diameter. 2) The skin tests require a fair amount of the time and cooperation of the patients, both for inoculation and for reading. 3) The test procedure could produce sensitization to the antigens. However, in the skin tests with tumor extracts, we have not seen any evidence for this.

Further studies, particularly comparative studies with other assays of cellular immunity, will be needed before the role of skin testing can be defined more clearly.

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Demonstration of Tumor-Associated Delayed Cutaneous Hypersensitivity Reactions in Patients With Lung Cancer and in Patients With Carcinoma Of the Cervix [1, 2]

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SUMMARY—A total of 75 patients with lung cancer and 25 patients with benign lung disease were evaluated for cutaneous reactivity to 3 standard microbial antigens and to 2,4-dinitrochlorobenzene (DNCB). Of the patients in each group, >95% reacted positively to at least 1 of the standard microbial antigens. Conversely, reactivity to DNCB was markedly depressed in the 75 cancer patients, especially so in those with nonresectable lesions. Sixteen of the cancer patients were tested with membrane extracts (ME) of autologous or allogeneic tumor cells, leukocytes, and normal lung. Nine patients developed delayed cutaneous hypersensitivity reactions (DCHR) to tumor ME (7 to autologous preparations and 2 to allogeneic preparations). Of the 15 patients tested, 4 reacted to leukocyte ME but only 1 of 14 tested reacted to ME of normal lung. Seven lung cancer patients developed DCHR to ME of fetal lung but not to fetal liver. Fourteen patients with carcinoma of the uterine cervix were tested with autologous and allogeneic ME of cervical carcinoma cells. Both preparations elicited DCHR, and there was a high incidence of cross-reactivity, suggesting the presence of a common antigen in the tumor cells of patients with cervical carcinoma.—Natl Cancer Inst Monogr 37: 197–203, 1973.

THE FACT that oncogenesis is more common in an environment deficient in cell-mediated immunity is well known, but the exact mechanisms allowing for malignant cell outgrowth are obscure. A depression of cutaneous reactivity to standard skin test antigens has been repeatedly demonstrated in patients with all stages of Hodgkin's disease; but, in patients with carcinoma, anergy seems to be confined mostly to those patients with advanced disease (*1, 2*), where general debilitation problably accounts for the decreased immunologic reactivity. In the study of Eilber and Morton (*3*), in which patients were sensitized topically to 2,4-dinitrochlorobenzene (DNCB), an antigen to which they had not been previously exposed, cancer patients with solid tumors reacted much less frequently (64%) than did normal controls (95%). Furthermore, the cancer patient's ability to be sensitized to DNCB was associated with a favorable prognosis; in nonreactive patients the prognosis was poor.

Tumor-associated immune mechanisms in man have most often been demonstrated by in

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vitro techniques utilizing immune lymphocytes or serum antibody and tumor target cells. Tumor immunity has also been demonstrated in vivo by the host's rejection of live tumor cells (*4, 5*) and by the elicitation of delayed cutaneous hypersensitivity reactions (DCHR) to tumor cell extracts. Utilizing this last technique, Hughes and Lytton (*6*), Stewart (*7*), and Oren and Herberman (*8*) demonstrated cutaneous reactivity to autologous tumor cell extracts in 25–50% of cancer patients. The importance of using only sterile tissue extracts of low protein concentration and of histologically confirming clinically positive skin reactions has been recently stressed (*7, 8*).

We have studied 100 patients with operable lung disease, 75 with cancer and 25 with benign lesions. We have also evaluated 14 patients with carcinoma of the uterine cervix. This report presents findings on host cutaneous reactivity to standard skin test antigens, to DNCB, and to membrane extracts (ME) of control and tumor tissues.

SUBJECTS AND METHODS

Patients

Part of this study involved 100 thoracic surgery patients, most of whom were from the Division of Thoracic and Cardiovascular Surgery at the George Washington University Hospital. Sixty-seven patients had primary lung cancer: 43 with squamous cell carcinoma, 20 with adenocarcinoma, and 4 with tumors of other histologic types. In 8 patients, cancer had metastasized to the lung from other organs. The 25 control patients had undergone thoracotomy for nonmalignant disease. The average age of the patients with benign disease was 39.5 years while that of the patients with malignancies was 57.8 years. All patients tested were ambulatory, and none had lost >10% of their body weight. None were skin-tested earlier than 2 weeks after surgery nor had received X-ray therapy or chemotherapy for 2 months before skin testing.

In addition, 14 patients with carcinoma of the uterine cervix were evaluated in the Surgery Branch of the National Cancer Institute.

Test Antigens

Standard microbial antigens.—All patients were tested intradermally with 0.1 ml each of 3 standard antigens: mumps (Eli Lilly & Co., Indianapolis, Ind.), *Candida* (Dermatophytin "O" 1:100, Hollister-Stier Laboratories, Spokane, Wash.), and streptokinase/streptodornase (Varidase 40 U/10 U/ml, Lederle Laboratories, Pearl River, N.Y.). Tests were read at 48 hours by measurement of perpendicular diameters with Vernier calipers. Induration >5 mm in average diameter was considered positive.

DNCB.—All of the thoracic patients were sensitized to DNCB by a modification (*9*) of the technique described by Eilber and Morton (*3*). A total of 2000 and 50 μg was applied topically at respective sites on the upper inner arm and the volar surface of the forearm. After 14 days, the sites were evaluated. If a spontaneous flare of induration and redness was present at both test sites, the patient's reaction was called 4+; if only the 2000-μg site reacted, the reaction was called 3+. If no reactivity occurred, the patient was challenged with 50 μg of DNCB at a separate site on the forearm and evaluated at 48 yours. If redness and induration at the challenge site were marked, the reaction was called 2+; if the reaction was weak, it was called 1+. When none of the test sites developed induration, the patient was called DNCB negative.

Preparation of Membrane Extracts

Sterile lung tumors were obtained from the operating room at George Washington University Hospital. A specimen of normal lung distant from the cancer and 60–100 ml heparinized blood were also obtained. Leukocytes were separated from whole blood in 2% methyl cellulose as described by Burger and Vetto (*10*). The lungs and livers of two 16-week human fetuses were the sources of fetal antigens. Sterile specimens of uterine cervix carcinoma were obtained from the operating room at the National Institutes of Health. Membrane extracts (ME) of tumor cells and control tissues were prepared by freeze-thawing and low ionic strength extraction, as described by Davies (*11*) and Mann

et al. (*12*) and modified by Oren and Herberman (*8*). A crude membrane pellet, obtained by centrifugation of the pooled extracts at 105,000 × *g* for 1 hour, was resuspended in saline and used for skin testing. Protein concentration of the ME was measured by the method of Lowry et al. (*13*). All test ME were adjusted to a protein concentration of 2.5 mg/ml, and each test injection contained 0.1 ml or 0.25 mg of protein. Sterility of the final extracts was tested by culture on blood agar plates and in thioglycollate broth. Only sterile ME were used. ME used for allogeneic testing were negative for the presence of hepatitis-associated antigen as tested by counter-electrophoresis (*14*). All ME skin tests were read at 48 hours, and most positive skin test sites were biopsied with a 2-mm skin drill, fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin.

RESULTS

Lung Cancer Patients

A total of 24 of 25 patients with benign lung disease and 73 of 75 patients with cancer reacted positively to at least 1 of the 3 standard skin test antigens. Quantitatively and qualitatively there was no evidence of less reactivity in the cancer patients.

Conversely, when sensitization to DNCB was evaluated, reactivity clearly differed between patients with benign and malignant disease and between patients with resectable and nonresectable disease (table 1). The χ^2 test for independence (*15*) was highly significant ($P<0.005$), showing that the proportions of responses in different categories differed with patient type. When the groups were tested in pairs, there was a significant difference between controls and patients with resectable disease ($P<0.025$) and between patients with resectable disease and those with nonresectable disease ($P<0.05$). Classifying the patients in 2×2 tables simply on the basis of response or no response (table 2), we found that the proportion of nonresponders was significantly higher among patients with resectable disease than among controls ($P<0.01$) and significantly higher among patients with nonresectable disease than among those with resectable disease ($P<0.01$, χ^2 analysis). Also grouping patients according to the histologic type of tumor, we found that the proportion of nonresponders was significantly greater among the patients with squamous cell carcinoma (22/43) than among those with adenocarcinoma (5/20) ($P<0.001$, χ^2 analysis). Nonreactivity to DNCB was not a function of age, since the proportion of nonresponders was approximately the same in each of the decades from 30 to 80.

The lung cancer patients' reactivity to control and tumor ME is shown in table 3. Of 14 patients tested with autologous ME, 7 developed DCHR to their tumor. Of these 7, 2 reacted to

TABLE 1.—*Numbers of patients in each of 3 categories classified according to response to DNCB**

Patient type		0	1+	2+	3+	4+	Total
Controls	f	1	3	12	8	6	25
	F	9.25	2.5	6.5	3	3.75	
	f-F	−8.25	0.5	5.5	0	2.25	
Patients with resectable disease	f	23	6	13	9	8	59
	F	21.83	5.9	15.34	7.08	8.85	
	f-F	1.17	0.1	−2.34	1.92	−0.85	
Patients with non-resectable disease	f	13	1	1	0	1	16
	F	5.92	1.6	4.16	1.92	2.40	
	f-F	7.08	−0.6	−3.16	−1.92	−1.40	
Total		37	10	26	12	15	100

*The observed frequencies f of controls, patients with resectable disease, and patients with nonresectable disease falling in each of the categories 0, 1+, 2+, 3+, and 4+ along with the expected frequencies F in each cell based on the assumption of independence of rows and columns.

TABLE 2.—*Evaluation of patients on the basis of response or no response to DNCB*

	Negative	Positive	Total
Controls	1	24	25
Patients with resectable disease	23	36	59
	24	60	84
Patients with resectable disease	23	36	59
Patients with nonresectable disease	13	3	16
	36	39	75

leukocyte ME and 1 reacted to his normal lung ME. Two patients tested with allogeneic ME reacted to tumor but not to normal lung. One of the two reacted to the leukocyte ME. Reactions to autologous tumor ME were positive in patients with squamous cell carcinoma and in those with adenocarcinoma. Of 11 lung cancer patients tested with fetal lung ME, 7 reacted positively. No patients reacted to ME of fetal liver. DCHR were positive to the lung ME prepared from each of the 2 fetuses. There was some cross-reactivity, since 3 cancer patients reacted positively to both tumor ME and fetal lung ME. However, 3 patients reacted only to tumor ME and 4 reacted only to fetal lung ME. In 10 additional patients with either benign lung disease or with cancer involving other organs, DCHR was positive to neither fetal lung nor liver ME.

Biopsies of positive skin test sites, including those of fetal and leukocyte ME injections, revealed mononuclear perivascular infiltrates compatible with delayed-hypersensitivity reactions.

Cervix Carcinoma Patients

Only 1 of 14 patients was anergic to all 3 of the standard skin test antigens (table 4). Six patients were tested against ME of their own tumor and leukocytes: 4 of the 6 reacted positively to tumor ME, and 1 of these 4 also reacted to autologous leukocyte ME. Of the 2 patients nonreactive to tumor ME, 1 reacted positively to leukocyte ME. Two patients (E. Jo. and E.Ja.) had large tumors, and ME prepared from these and paired leukocytes were used for allogeneic testing in 13 patients. No patient reacted to either allogeneic leukocyte ME, but 8 of 13 had positive DCHR to at least one of the tumor ME and 3 patients reacted to both. Of the 4 patients reacting to autologous tumor ME, 3 also reacted to one or both of the allogeneic tumor ME. Histologic examination of the positive biopsy sites was characterized by findings consistent with a delayed cutaneous hypersensitivity reaction.

DISCUSSION

Cutaneous anergy to standard microbial antigens has been well documented in patients with

TABLE 3.—*Delayed skin reactivity to tumor, normal, and fetal tissue ME in patients with lung cancer*

Patient	Common antigens	Tumor	Normal lung	Leukocyte	Fetal lung	Fetal liver
C.S.	+	–	–	NT*	NT	NT
R.D.	+	–	–	–	+	NT
J.N.	+	+	–	–	–	–
J.H.	+	+	NT	–	+	–
C.H.	+	–	–	–	+	NT
M.S.	+	+	+	+	–	–
J.S.	+	–	–	–	–	–
E.K.	+	+	–	+	NT	NT
J.G.	+	+†	–†	+†	+	–
O.C.	+	–	–	–	NT	NT
V.V.	+	+	–	–	NT	NT
R.E.	+	+	–	–	NT	NT
E.F.	+	–	–	+	+	–
E.T.	+	+	–	–	+	–
E.Z.	+	–	NT	–	+	–
H.H.	+	+†	–†	–†	–	–

*NT = not tested.
†Denotes reactivity to allogeneic preparation.

TABLE 4.—*Delayed skin reactivity to tumor and normal tissue ME in patients with carcinoma of the uterine cervix*

Patient	Common antigens	Autologous tissue		Allogeneic tissue	
		Tumor	Leukocyte	Tumor	Leukocyte
M.H.	+	+	−	+*	−*
F.C.	+	NT†		+	−
A.Q.	+	NT		+	−
N.R.	+	−	+	+*	−*
E.Jo.	+	+	−	NT	
E.Ja.	+	+	−	+	−
C.B.	+	NT		−	−
A.B.	+	NT		−*	−*
F.H.	+	NT		−	−
D.A.	+	+	+	+	−
I.W.	+	−	−	+*	−*
L.D.	+	NT		−	−
Y.J.	+	NT		+	−
A.B.	−	NT		−	−

*Denotes reaction to 2 allogeneic preparations.
†NT = not tested.

Hodgkin's disease (*16*), but to what extent it occurs in patients with carcinoma is controversial. Solowey and Rapaport (*2*) studied 91 patients with carcinoma and noted that anergy was associated with metastatic disease. Lamb et al. (*1*) found that debilitated carcinoma patients were anergic to microbial antigens while those in "good condition" had little or no depression of reactivity. Krant et al. (*17*) found a markedly decreased cutaneous reactivity to tuberculin in patients with bronchogenic cancer. Many of their patients, however, were receiving X-ray therapy or chemotherapy or were in a terminal state. In the present study, the exclusion of debilitated patients and patients receiving adjuvant therapy perhaps accounts for our finding no decreased cutaneous reactivity to standard microbial antigens in the lung cancer group.

When compared to control patients with benign lung disease; lung cancer patients had markedly depressed reactivity to DNCB. This was especially true in those with nonresectable lesions where only 3 of 16 patients were able to be sensitized. Furthermore, there were significantly more nonresponders among squamous cell carcinoma patients than among adenocarcinoma patients. We have not followed all of our patients long enough to correlate DNCB response with prognosis, but the high incidence of nonresponsiveness in those patients with nonresectable lesions agrees with the findings of Eilber and Morton (*3*) who noted a positive correlation between poor prognosis and negative DNCB response in patients with other types of solid tumors.

The cancer patients had probably been sensitized to the standard microbial antigens early in life, and the reactivity to these antigens suggests an intact efferent limb of the immune reponse. The same patients' inability to be sensitized to a new antigen (DNCB) suggests a faulty afferent limb of immunity, though the exact site (central, or efferent) of the defect and its temporal relationship to oncogenesis are unknown.

The development of delayed cutaneous hypersensitivity reactions to autologous tumor ME elicited in 7 of 14 patients with lung cancer and 4 of 6 patients with cervical carcinoma supports the concept of host cellular immunity to tumor cell antigens. The relatively high incidence of cutaneous reactivity to autologous leukocyte ME was unexpected, since the preparations were sterile and the protein concentration of the injected extracts was low. Oren and Herberman (*8*) found that 10% of normal individuals had positive cutaneous reactivity to autologous leukocyte ME when the protein concentration was 0.33 mg/0.1 ml or less. They postulated that the reactivity could be due to the presence of delayed-hypersensitivity mediators in the leukocyte ME. Lymphocytes have been shown to be the principal cells which elaborate

the mediator substances (*18*); however, migratory inhibitory factor (*19*) and leukotactic factor (*18*) are soluble proteins which would be expected to remain in the supernatant after the 105,000 × *g* centrifugation. The fact that reactivity rarely occurred with ME of autologous normal lung tissue perhaps indicates the unique properties of the leukocyte preparation used. This could be related either to the presence of delayed-hypersensitivity mediators associated with pelleted lymphocyte ME or to substances released from polymorphonuclear leukocytes which were present at the beginning of the extraction procedure. Reactivity of cervical carcinoma patients to tumor ME could not be similarly controlled because normal autologous cervical tissue was not obtainable at surgery.

Because of the relatively small amount of protein present in the lung tumor ME, single allogeneic preparations could not be used for testing large numbers of patients. Two patients did, however, give positive reactions to separate allogeneic tumor preparations. In patients with carcinoma of the cervix, cross-reactivity was readily demonstrated against 2 allogeneic tumor preparations. Patients reacting to their autologous tumor ME also reacted to allogeneic tumor preparations. This suggests a common antigenicity in the tumor cells of patients with cervical carcinoma.

The high degree of reactivity in the lung carcinoma patients to fetal lung ME has not been previously demonstrated and possibly represents the presence of an antigen(s) in fetal lung tissue which is immunologically similar to an antigen(s) of lung carcinoma cells. Although the number of patients tested was small, the reaction seemed specific in that the lung cancer patients reacted to lung ME prepared separately from 2 fetuses but did not react to paired fetal liver ME. Furthermore, patients with benign pulmonary disease and patients with cancers involving other organs did not react to ME of either fetal lung or liver. The incidence of cross-reactivity was about 25%, since 3 of 11 patients reacting positively to fetal lung ME also reacted to tumor cell ME. Hollinshead et al. (*20*) previously showed that patients with carcinoma of the large bowel developed DCHR not only to soluble fractions of autologous tumor cells but also to soluble antigens prepared from the digestive tracts of first and second trimester fetuses.

We are in the process of using the fetal ME to test a large number of lung cancer patients and patients with cancers of other organs to delineate more clearly the frequency and specificity of this reactivity.

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PART IV

SKIN TESTS

DISCUSSION

OUTLINE OF DISCUSSION—SKIN TESTS

I. Antigens used
   - A. Whole cells versus antigen extracts
   - B. Differences between various extracts
   - C. Purification of skin-reactive antigens
   - D. Effect of protein concentration
   - E. Controls against bacterial contamination
   - F. Normal cell controls used
   - G. Allogeneic extracts—same tumor type, different tumor type

II. Recipients
   - A. General cellular reactivity of patients: ? anergic
   - B. Relationship of test results to drug therapy, surgery, and stage of disease
   - C. Control patients—for specificity tests

R. Herberman: Before we begin the discussion, Dr. Hollinshead has some interesting data to present.

A. Hollinshead: I would like to suggest the usefulness of highly specific, absorbed antisera in the various in vitro tests. For 4–5 years we have been studying the soluble membrane and membrane antigens of vaginal, vulva, and cervical cancers and normal counterpart tissues and, in skin tests, have found a specific response to the membranes from the cancer tissues. When we separated the membranes from the pellets, then further subjected the membranes to low-frequency sonic disruption at different intervals, and isolated these membranes over Sephadex G200, we found that the soluble portion that reacted was in the molecular weight range of 45,000–145,000. The pellet of membrane remaining was negative. We therefore continued to study the soluble component.

A carefully prepared, absorbed, highly specific herpes type-2 antiserum reacts in complement-fixation (CF) tests mainly with this partially separated, soluble fraction from various genital cancer cell membranes and not with other cancer fractions nor with control fractions of normal vaginal tissue (table 1). In a coded, double-blind test, Dr. Melnick and Dr. Rawls of Houston, Texas, tested and retested the cervical, vulva, and vaginal Sephadex fractions with matched cancer and control sera.[1] Of 21 sera from women with cervical cancer, 20 reacted with this soluble membrane antigen, which also was given in the skin test. The reaction also occurred in 5 of 21 control matched sera. There was only a prevalence of reaction here, but neither cancer nor control sera reacted with similar preparations of normal vaginal tissues, and the antibody does seem to be more prevalent in the cancer population. But one thing that must be pointed out is that these are the prostitute sera, with an average of 7 pregnancies each. In addition, the sera were not absorbed. There would have been no basis for comparison if the specific sera had not been used first. We are continuing this study with other types of specific sera and with absorbed matched human sera.

We also conducted skin tests in young-adult, female albino guinea pigs (Hartley strain) weighing about 400 g (obtained from Hazelton, Inc.). Found to be free of herpes-like virus, these animals were considered ideal for our experiments. Groups of these guinea pigs were sensitized to purified herpesvirus types 1 and 2, as well as to uninfected HEK cell material purified in the same manner as the viruses. The backs of the guinea pigs were shaved gently with animal clippers and several scalpel blades. Each of the test materials (0.1 ml) was injected intradermally on the left and right side of the spine with a tuberculin syringe, with the bevel of the needle pressed in a slanted position against the skin. The animals were observed for initial and delayed reactions at 1, 12, 24, 48, and 72 hours afterward. Both erythema and induration were measured, and the diameter was recorded in millimeters. Biopsies were taken for pathologic evaluation of delayed-hypersensitive reactions.

We tested 3 guinea pigs sensitized to herpesvirus type 2 with freshly prepared antigens and, further, we tested these same materials in 3 guinea pigs sensitized to herpesvirus type 1 and in two control guinea pigs sensitized to an HEK cell preparation prepared in the same way. Animals were cross-challenged with membrane and soluble membrane antigens of equivalent protein content from a cervical tumor and from normal vaginal cells from the same patient. Normal skin cell membranes from the same patient, another DNA virus, adenovirus type 12 (Huie strain), and similar soluble membrane fractions from breast tumor cells were included as controls. Table 2 summarizes the results of tests in the virus-sensitized

[1] Hollinshead A, Lee O, McKelway W, et al: Reactivity between herpesvirus type 2-related soluble cervical tumor cell membrane antigens and matched cancer and control sera. *In* International Virology (Melnick JL, ed.), vol 2. New York, S. Karger, 1971, p 105. *See also* Proc Soc Exp Biol Med, 1972. In press.

TABLE 1.—*Cutaneous hypersensitivity reactions to autochthonous tumor and control tissue membranes and soluble membrane fractions in patients with cervical, vulva, and vaginal cancer*

Patient No.	Delayed-hypersensitivity reactions*	CF†
1. *Cervical tumor*		
Soluble fraction I	–	0
Soluble fraction II	+	1:64
Soluble fraction III	–	0
Soluble fraction IV	–	0
Control tissue		
Soluble fraction I	–	0
Soluble fraction II	–	0
Soluble fraction III	–	0
Soluble fraction IV	–	0
2. *Cervical tumor*		
Membranes	+	NT‡
Soluble fraction I	–	0
Soluble fraction II	+	1:16
Soluble fraction III	+	1:4
Soluble fraction IV	–	0
Control tissue		
Membranes	–	NT
Soluble fraction I	–	0
Soluble fraction II	–	0
Soluble fraction III	–	0
Soluble fraction IV	–	0
3. *Vulva tumor*		
Soluble fraction I	–	0
Soluble fraction II	+	1:16
Soluble fraction III	–	0
Soluble fraction IV	–	0
Control tissue		
Soluble fraction I–IV	–	0
4. *Vulva tumor*		
Membranes	+	NT
Soluble fraction II	+	1:8
Soluble fraction III	–	0
Control tissue		
Membranes	–	NT
Soluble fraction II	–	0
Soluble fraction III	–	0
5. *Vaginal tumor*		
Membranes	+	NT
Soluble fraction II	+	1:32
Soluble fraction III	–	1:4
Soluble fraction I+IV	–	0
Control tissue		
Membranes	–	0
Soluble fraction I–IV	–	0

*Induration of ≥5mm at 24–48 hours is considered positive. Er thema varies, tends to decrease in intensity, and is unrelated to pathologic evaluations of biopsied material for true delayed-hypersensitivity reactions.

†CF titer as measured with herpesvirus type-2 hyperimmune guinea-pig sera (1:20). A zero means <1:2. Complement and cell antigen controls were negative.

‡NT = not tested.

guinea pigs; reactions in the 2 control guinea pigs did not last longer than 12 hours, except for a 24-hour delayed response to the normal skin membranes. Herpesvirus type 1 produced a delayed-hypersensitivity reaction in both the guinea pigs sensitized to herpesvirus type 2 and in those sensitized to herpesvirus type 1, whereas herpesvirus type 2 seemed to be more specific. Of considerable interest was a 24-hour delayed response to normal skin membranes, and we continue to consider the role of other antigens in the production of a delayed-hypersensitivity response. However, neither the membranes and soluble fractions from normal vagina nor the soluble fractions from breast tumor produced delayed cutaneous responses. The specific 48-hour delayed response to cervical cancer cell membranes and to soluble fraction II in guinea pigs sensitized to herpesvirus type 2 and the lack of response in guinea pigs sensitized to herpesvirus type 1 suggested that further study of these soluble antigens was important.

We further separated the reactive soluble cancer frac-

TABLE 2.—*Delayed-hypersensitivity reactions* of guinea pigs inoculated with soluble fractions of tumors and viral antigens*

Antigen to which animal is immune	Test antigen												
	Cancer of cervix				Normal vagina			Breast cancer		Skin			
	Membrane†	I+IV‡	II‡	III‡	Membrane	II	III	II	III	Membrane	HSV-1	HSV-2	Adenovirus 12
HSV2 (3 animals)	+	–	+	–	–	–	–	–	–	+	+	+	–
HSV1 (3 animals)	–	–	–	–	–	–	–	–	–	±	+	–	–

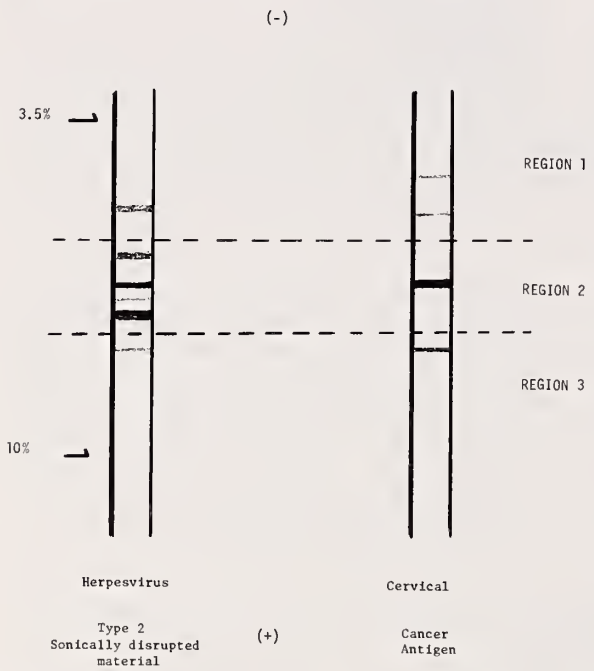
*24-hour and 48-hour induration of ≥5mm is considered positive.

†Membrane—crude membrane antigens.

‡Soluble separated membrane fraction: I and IV were combined; II and III were tested separately.

tion II by gradient polyacrylamide gel electrophoresis.[2] In the lower region 3 (text-fig. 1) there is a band similar to the herpes material sonically disrupted. Of interest is that the antisera also contain antibodies to the herpes type 2 nonvirion antigens, and we are not sure if this is a herpes antigen or one that is induced. These 3 regions having been twice eluted are being tested in a series of cervical cancer patients. Dr. Herberman and Dr. Jaffurs are helping with this study to see whether the skin test antigens are in different regions or are in the same region. In the coded blind CF tests done with Dr. Melnick and Dr. Rawls, the section 3-eluted material was tested with serial dilutions of cancer and matched control sera; we found that, with the more purified material, we could titrate the positive reactions with the cancer sera.[2] I believe this illustrates the possible usefulness of highly specific sera for other types of in vitro studies and a way to correlate such tests with in vivo studies.

Dr. Herberman, Dr. T. Stewart, and I tested many cases of lung cancer in the last 3 years and found some



TEXT-FIGURE 1.—Cervical cancer soluble-membrane fraction II separated by Sephadex gel filtration and sonically disrupted material of herpesvirus type 2 (SAV strain) were fractionated by gradient polyacrylamide gel electrophoresis (3.5, 4.75, 7, and 10% stacked gel). A tracking dye (bromphenol blue) was used in each preparation. Gels were stained with Coomassie brilliant blue.

[2] HOLLINSHEAD A, MCWRIGHT C, ALFORD T, et al: Separation of skin-reactive intestinal cancer antigen from the carcinoembryonic antigens of Gold. Science 177:887–889, 1972.

responses to "normal" lung material from cancer patients. We have not gotten as much normal lung tissue from noncancerous patients as we would like, and we are trying to solve this problem. We find that most of the responses are to the cancer membranes. Unlike leukemia whole cells or membranes, we find that solid tumor cell membranes do not always produce a skin response. We also isolated specific antigens, which produce more pronounced skin reactions, for the different *types* of lung cancer and found great differences between the types. Dr. Stewart believes the true delayed-hypersensitivity reactions, as further assessed in the biopsies, are superior to those he saw with whole cells or membranes.

In reference to Dr. Mavligit's study with soluble KCl extracts, I've had limited experience with KCl extraction, but we did compare it with the sonication procedures at one point. There was a slight alteration in the structural arrangement of the antigen we studied; whereas the HL-A antigens were intact. So while KCl extraction is an excellent way to separate the HL-A antigens, it may or may not be, depending on the system, a good way to treat cancer cells for extraction of select antigens.

Herberman: A question was raised about the use of whole cells versus antigen extracts for skin testing. We have had very limited experience with such a comparison. We tested 5 acute lymphocytic leukemia (ALL) patients with both autologous membrane extracts and whole blast cells (1×10^6 cells); with 3 of the 5 patients, we got a positive test with both the blast cells and the membranes. With the other 2 patients, both of their tests were negative. We believed at that point that the reactivity was relatively comparable, and we have elected to test with a nonviable extract. We thought this was particularly preferable for patients in remission. We have been concerned about the problem of giving viable cells to patients in remission because of the possibility of reinducing the disease from the injected cells. There is also the possibility of reinoculation of a virus that is directly related to the disease. By electron microscopy of the membrane extracts, we have never seen any virus. Also, membrane extracts prepared from a murine leukemia induced by Rauscher virus did not contain detectable infectious virus.

J. Holland: Did all 5 of your patients have relapses? Your proposition loses its meaning unless all 5 of your patients remained in remission, since the cure of the disease is really variable.

Herberman: This is just a concern that we had. The 2 patients with negative skin tests were in relapse at the time of testing. The 3 patients with positive tests remained in remission for over 6 months. There was no growth of a tumor nodule at the site of inoculation.

J. Gutterman: We skin-tested 8 patients with both the whole leukemia cells at a concentration from 10^4 to 10^6 as well as with the soluble KCl antigen. Four of those patients were anergic to a battery of standard antigens, and they were nonreactive to both the cells and the antigen. However, of the 4 patients positive to at least

1 of the standard antigens, 4 did not react to the whole tumor cells, but 3 of the 4 did exhibit positive reactivity to the soluble antigen. A biopsy was performed on 2 of these positive reactions, and both had histologic evidence of delayed hypersensitivity. So we do see some disparity between the antigen and the whole cells. In addition, concurrent with our serial blastogenesis studies in 35 patients, we have routinely skin-tested with the whole cells and we have never seen a positive reaction with 10^6 cells.

Herberman: What was the equivalent in whole cells of the amount of KCl extract needed to produce positive reactions?

Gutterman: Generally we get about 10 μg/ml of antigen equivalent to 10^6 cells and our positive skin test has been around 100–300 μg.

G. Santos: And your whole cell testing was with what amount?

Gutterman: In a dose range of half-log increments from 10^4 to 10^6.

Santos: So then below your KCl equivalent?

Gutterman: Yes, but they are not equivalent. However, we have immunized 10 patients with 2×10^7 whole cells at 5 different locations. Of those 10 patients, 1 patient has been skin-test positive at the site of immunization.

Herberman: It is probably fair to say then that you do not have a complete comparison. You're working in one case with smaller amounts of antigen. The skin reactions we have seen with the insoluble membrane extracts have been rather subtle, generally in the range of a 5–8 mm, shallow induration. On the other hand, soluble preparations produced by sonication and separated on Sephadex generally give a bigger reaction and are easier to read. The reason for that is not clear. Is it possible that the whole cells produced small positive reactions that may have been overlooked?

Gutterman: With whole cells, we have rarely seen induration at 24–48 hours. With our antigens, we usually get a clear-cut reaction.

S. Golub: I wonder if any of the people trying skin reactions with soluble antigens have attempted to use the Rebück skin window? This might be a way to quantitate fairly small reactions.

Herberman: The only one I am aware of who has used the Rebück skin-window technique is Dr. M. Black. He has put thin sections of breast cancer on areas of abraded skin.

Golub: It might be worth trying to use soluble antigens.

Herberman: With Dr. Rosenberg, using the direct migrating system he described earlier in this Conference, we studied about 5 patients with the membrane extracts that had been used for skin testing. In 2 solid tumor patients, the extracts produced positive skin tests and also inhibited migration. The extracts clearly inhibited the patients' cells more than the lymphocytes of the normal controls. In addition, one of our identical twin patients with acute myelogenous leukemia (AML) was studied by both the skin test and by migration inhibition. Extracts were used from the leukemic patients' blast cells and from the normal leukocytes of the normal twin. Migration was inhibited only by the tumor cell extract.

C. Alford: I tested 4 patients with a malignant melanoma cell line at concentrations of 10^6–10^7. None of the 4 reacted to the whole irradiated cells. The same amount of cells were sonically disrupted and separated, and 2 of the patients were tested with the membranes and soluble fractions. They did not respond to the membrane but they did respond to the soluble fraction.

Herberman: This has been the general experience with melanoma, hasn't it? The membranes have given less reactivity than the further-separated, soluble fractions.

R. Oldham: Are you talking about the soluble fraction from the tissue culture cells? Were the patients positive to extracts from melanoma tissue?

Alford: Yes, they were. They, again, were positive to their soluble fractions, but not to their membranes of the original tumors.

P. Levine: In studies with Dr. Herberman and others, we have been trying to look at the reactivity of lymphoma patients to extracts from their own tumors and various cell lines. American patients with Burkitt's lymphoma and Hodgkin's disease frequently have elevated antibody titers against the Epstein-Barr virus (EBV), and we have tried to compare their humoral activity to EBV with the cell-mediated immunity to EBV-associated antigens and possible tumor-associated antigens. The cell lines used in the skin testing were monitored by immunofluorescence with the use of Dr. Klein's serologic reagents and testing procedures for blocking activity, so we could tell how many EBV cell-associated surface antigens were present on a specific cell line before we made a membrane preparation for skin testing. I would like to emphasize that, in some of these patients, tested serially, particularly the Hodgkin's disease patients, there has been an obvious change in general reactivity which has been difficult to quantitate, but must be taken into account in evaluating the reactions against the cell lines. Two children had sloughing or major delayed-hypersensitivity reactions on initial testing with streptokinase/streptodornase or Candida, and on retesting they were still "positive," but clearly less reactive than on the first test. You may find that there is a change in your standard tests with the course of the disease, and you might want to express your results as a ratio of nonspecific/specific rather than just describing positives.

Herberman: The issue that Dr. Levine is bringing up is important. My own feeling is that skin tests are not very quantitative. It is hard to assess accurately the degree of reactivity when you are measuring only 2 dimensions; you cannot really measure the depth of induration. The best way of trying to quantitate these reactions is to do dilution studies with some of the extracts. This was the principle employed with tuberculin. One could detect the very reactive population by testing with very low concentrations of the antigen. The problem with this is the requirement for a large number of tests.

D. Rubin: Did you test your membranes for the presence of EBV-associated antigens by immunofluorescence? Was the membrane preparation itself positive, the same way that the tumor was positive?

Levine: No, we do not have a fluorescent technique that can monitor the final membrane preparation, since the antigen used is soluble.

Rubin: Is the extract membrane? Otherwise, you don't know if you're testing the same thing.

Levine: We do absorption, gel diffusion, and other studies with these preparations. It should also be emphasized that *every* lymphoid cell line has the EBV genome so, whether you detect EBV-associated artigens or not, anybody who uses a lymphoid cell line for skin testing has to be aware that there is EBV in the cell line. This may well complicate any immunologic studies in which tissue-culture cell lines are used.

Rubin: That is precisely the point. Even if there is an EBV antigen expressed in the cell line, you have to know in what form it is expressed before you can start comparing in vitro with in vivo testing for the antigens.

Hollinshead: I've done a small study with Dr. Herberman that may be helpful.[3] We looked for complement-fixing antigens on the surface. We were able to separate the HL-A component and the EBV CF-reactive component away from the skin test component, and it appears to be separate. But this is going to have to be done on a larger scale, Dr. Levine, to know whether it is valid.

Herberman: One of the questions raised earlier was the effect of freezing on the skin test antigens. From our own experience, freezing of the membrane extracts hasn't appeared to affect reactivity over a period of 3 years. I know Dr. Hollinshead has an impression that the soluble antigens may deteriorate upon freezing. In regard to the leukemia cells, we have made extracts from dimethyl sulfoxide (DMSO)-frozen cells, and this has given us some positive results, although it is much harder to make a good extract from cells stored in that way. The yields are generally poor. Does anybody else have any information about frozen cells versus entirely fresh cells?

S. Wells: We have found that freezing solid tumor cells does not seem to affect reactivity. We have no experience with DMSO-frozen cells. When we have the final membrane extract to be used for skin testing, we freeze 0.1 ml portions in tuberculin syringes. This prevents repeated freezing and thawing of a large membrane extract preparation.

Santos: I could see why one would like to use extracts if these really were purified. We heard some data that run a little counter to the data obtained from guinea pig investigations, but these data obviously need more confirmation. With the amount of cell equivalents you need for those extracts, you are really throwing away a lot of cells if whole cells will work. If you're going to collect cells, particularly from leukemia patients, you can put away whole cells and actually get more mileage if you want to find out what happens during the treatment course of a patient. Maybe I'm being provocative on purpose; I don't want to start doing membrane studies right now and I'd like to know if there is a reason that I should.

Herberman: All I can say at the moment is we have demonstrated that the membrane studies work. You have not yet demonstrated that testing with whole cells will work as a method of following the patient.

W. J. Martin: It would seem necessary to exclude a nonspecific inflammation resulting from the inoculation of tumor cells or their extracts. A suitable control would be to administer the test material to normal subjects. This approach, however, with its inherent risks would be hard to justify.

Santos: What do you mean a normal control? I would give them no extract of leukemia at all, because if 10 years from now they get colon carcinoma and you try to convince them that you did not give them some virus, you won't succeed.

Martin: I agree with you wholeheartedly. Yet, if you cannot include that control, of what significance is a positive reaction in a tumor-bearing host?

Herberman: That is a good question. Maybe we should address ourselves to the kinds of controls that one can do. I believe quite strongly that, until more information is obtained, we should not inoculate materials from cancer patients, even in soluble form, into individuals without cancer. There are a series of controls that can be done, though, that give some information, although not complete, about the specificity. One of the best available controls is to test the extract prepared from one type of cancer in patients with other types of cancer. In these patients, you are not as concerned about inducing other diseases because they already have cancer. These tests have given indications of specificity; e.g., extracts made from ALL blasts do not give positive reactions in patients with AML or in patients with solid tumors. This provides some information on the specificity of the very extract that is producing a positive result. Another type of control, which is quite important, is to test with extracts of normal tissue. However, there are a couple of problems here. First, what normal tissue should one use as a control? Can one really get the appropriate cells in equivalent amounts so that the control is adequate? In ALL, one can get fairly close—one can use normal lymphocytes. However, with most of the solid tumors, e.g., with breast cancer, it is very difficult. If one used breast tissue from the cancer patient, it may contain some malignant cells. In both the "normal" tissues from the mastectomy specimen and in "normal" breast from a patient with cystic disease or some other benign disease, there may be relatively few epithelial cells. Even with this being an inadequate control, we have seen some positive reactivity. Perhaps if we had a really good source of normal epithelial cells from the

[3] Hollinshead AG, Herberman RB: Separation of the major histocompatibility antigens from other antigens present on human leukemic and white blood cell membranes. Fifth Int Sympos Comparative Leukemia Research. Padova, Italy, 1971. In press.

breast, we would get reactivity as strong as any of the tumors have produced.

Wells: We ran into the same kind of problem when testing lung cancer patients. We found that about 55% of our patients reacted to lung-cancer membrane extracts and about 25% reacted to leukocyte membrane extracts, whereas only 1 of 16 patients reacted to membrane extracts of normal lung tissue. We have not tested normal controls with tumor membrane extracts, although we have tested patients with cervical cancer with lung-tumor membrane extracts. We have tested normal controls with fetal antigen membrane extracts but this is a different situation.

Herberman: Despite the inadequacy of the available controls, they certainly need to be done. They give at least some information about specificity. We still have some hope that use of the migration inhibition assay will allow us to perform the controls that are difficult to justify in vivo.

I wanted to raise the question about allogeneic extracts. We've been having a rather surprising experience with the leukemia extracts. We thought we would see a great deal of reactivity against HL-A antigens, since they are in these extracts. One might expect that reactivity to HL-A antigens would present a major problem, particularly in a population of cancer patients who are given blood and/or platelet transfusions. On the contrary, however, we have seen very little activity of this type. Hardly any of the reactivity that we do see in transfused patients can be explained by reactivity to HL-A antigens.

Rubin: Do most patients with ALL react to allogeneic ALL extracts as well, or is the reactivity seen primarily in testing of autologous extracts?

Herberman: Less than half of the ALL patients have reacted to allogeneic ALL extracts. In addition, we usually have tested patients with 3 or 4 allogeneic preparations; patients who have been positive generally react to only 1 of the extracts. So, we have not seen much cross-reactivity. Dr. J. Ziegler, in recent studies in Burkitt's lymphoma patients in Uganda, has seen no convincing evidence of cross-reactivity to allogeneic extracts. Because of the possible role of EBV, one might particularly have expected wide cross-reactivity in this disease.

Santos: That causes considerable problems for a person who thinks that the etiologic agent is a virus.

G. Klein: Before drawing that conclusion, it would be important to see whether known virus-induced tumor antigens can show up in the skin tests. Have any of the known animal models, with cross-reactive antigens, been tested?

Herberman: That is a fair question. I am not aware of any models, with the possible exception of Dr. Hollinshead's, that have really been tested. Most of the skin testing in animal tumor systems has been done in guinea pigs, with chemically induced tumor systems where you would expect individual, specific antigens.

G. Klein: One can also make the point that Dr. Morton and Dr. Vaage have shown the existence of individually distinct antigens, in addition to virus-determined common antigens, in the mammary tumor virus-induced mammary carcinomas. Possibly similar individually distinct antigens are present in all systems. If so, I do not know whether one would expect the common or the individually distinct antigens to show up in the skin tests.

Herberman: Patients with intestinal cancer have a broad reactivity to tumor extracts. They react as well to allogenic extracts as to the autologous. Similarly with melanoma and breast cancer, the reactivity is about the same. With intestinal cancer, we raised the question of whether the antigen being detected was carcinoembryonic antigen (CEA), which might explain some cross-reactivity. With the other two, there is at least some indication that some of the antigens being detected are tissue specific.

G. Klein: Then you have made the point that the CEA could be separate from whatever is responsible for the delayed-hypersensitivity reaction in the carcinoma of rectum tissue?

Herberman: That's right. Although the skin-reactive antigen still has to be considered some type of CEA, in that the same antigen is found in fetal material as well as in the tumor, it seems to be a distinct antigen from the antigen Gold described in serologic tests.

G. Klein: Could it be that your membrane fractions do have CEA material until you purify them? Am I right in thinking that you found CEA in both of your preparations, but then you separated the CEA from the membranes and found that the membranes induced the skin reactions but not the CEA?

Herberman: Dr. Hollinshead has gone through the same purification as far as the acrylamide gel electrophoresis with fetal liver as well as with tumor: The skin-reactive Sephadex fractions of both contained CEA. On acrylamide gel, the top part of the gel from both the fetal and tumor material gave positive tests in Gold's assay for serologic CEA; the lower part gave positive skin reactions.

G. Klein: I am interested to hear that, because I understand that the existence of immunologic reactivity to CEA in patients is very much in question.

Rubin: I might mention some of our results of a few years ago on the efficacy of intradermal immunization with viable tumor cells. We were working with Rauscher and Moloney virus-induced ascites cell lines, which were shown to contain cross-reactive antigens by various investigators using in vitro tests. The surprising finding was that, whereas Rauscher virus or Rauscher virus-induced tumor cells inoculated into the skin protected against subsequent challenges of either Rauscher or Friend virus-induced tumors, Friend virus-induced tumor cells did not protect against challenge with either cell line. It's really a very complicated system, even when known cross-reactive antigens can be demonstrated. Intradermal immunization is an important approach in evaluating tumor cells of extracts for both the presence of cross-reacting antigens and the nature of the immune response elicited. The presence of known cross-reactivities,

demonstrated in certain in vitro tests, does not necessarily predict the nature of host-tumor interactions in vivo.

M. Sigel: We are speaking largely of matters of immunologically specific components of skin-reaction tests; yet all of us know that a skin reaction contains both specific and nonspecific effector substances. Thus discrepancies in comparing in vivo and in vitro results may be derived from nonimmunologic factors in addition to aberrations in the functions of immunologically relevant cells.

Herberman: I think the point you are making is well taken. A paper by Dr. S. Salmon's group last year in the *New England Journal of Medicine* made this point. They skin-tested their patients not only with recall antigens but also with croton oil, which was supposed to look at the general ability to cause inflammation in the skin. I believe there are 2 problems with this study. 1) Most of the patients were on chemotherapy at the time of skin testing, so they were not entirely representative of the tumor patients on whom we try to do the skin testing. 2) The original study on which they based their investigations does not entirely support their claim; there were a few patients who were skin-test positive to recall antigens yet were negative to croton oil. I agree with your general point but I'm not sure that, at the moment, there is a suitable agent to measure this.

W. Churchill: It also went the other way. There was 1 patient who was negative for the skin test but positive for the croton oil, because their thesis was that, if the inflammatory response was all right, they should be able to sensitize the patient.

Herberman: One last issue, in regard to the skin tests: the method to assess general reactivity of the patients. I think Dr. Wells might want to speak on this. Is sensitization to dinitrochlorobenzene (DNCB) better than recall antigens? If one tries to sensitize with DNCB, one is looking at the ability of the patient, at that point in time, to be sensitized by a new antigen and also to have a positive skin reaction after sensitization. Conceivably a tumor patient could have become sensitized to his tumor antigens a year or two earlier and still have the ability to react to those antigens, but with progression of the disease, have lost the ability to become sensitized to a new antigen.

Wells: You have to be careful about this. It takes 2 weeks to do DNCB testing, which can be impractical clinically. We initially tried to sensitize patients preoperatively and then challenge them postoperatively. This was unsatisfactory because a negative test result might have been due to anesthesia or operative stress. Then we started testing at least 2 or 3 weeks after the operation. We have seen patients who were DNCB negative and who still reacted to tumor antigens. The point you made was a good one. With DNCB you are presenting patients with a new antigen, and negative reactivity doesn't imply that they have always been negative nor does it imply that they will or will not react to tumor antigen extracts. If we had to choose between the common antigens and DNCB, I think we would take the DNCB as the indication of the general immunologic status of the patient. Almost everybody we have seen has been reactive to at least 1 of the 3 common antigens, and I'm sure you have found the same thing. We found that reactivity to DNCB is much less in nonresectable lung cancer patients than in resectable ones. We are following our lung cancer patients for 1 year, to find out if there is a correlation between negative DNCB response and poor prognosis. As you know Dr. F. Eilber and Dr. D. Morton found a striking correlation between negative DNCB response and poor prognosis.

Herberman: You have seen some positive reactions to tumor extracts in tumor patients who could not be sensitized to DNCB?

Wells: Yes.

E. Klein: In collaboration with Dr. G. Petranyi, we have immunized guinea pigs with cells from a human lymphoblastoid line. The skin test, lymphocyte transformation, migration inhibition, and cytotoxicity against both human and mouse cells were monitored. These tests using the draining lymph nodes became positive on the 4th day after immunization with maximal values on the 6th day; thereafter their strength declined. Thus there was a parallel in these tests. When mouse cells were used in the in vitro assays as antigen, the tests were negative. The skin test, however, was positive.

PART V

INTERRELATIONSHIPS BETWEEN ASSAYS

DISCUSSION

OUTLINE OF DISCUSSION—INTERRELATIONSHIPS BETWEEN ASSAYS

I. Are tests detecting same antigens?

II. Are tests measuring same phase of immune response?

III. Direct correlations between assays?

IV. What methods are available to dissect this out (e.g., effect of blocking sera from one assay on another; separation of reactive cells; inhibition by antigens).

R. Herberman: What are the relationships between the different assays? Have any 2 of these assays about which we have been talking really been shown to be measuring the same thing? To start the discussion, I'll recount the experience our group has had with acute leukemia patients, in collaborative studies with Dr. Halterman and Dr. Leventhal. There were 20 patients with acute leukemia studied by skin test, by ^{51}chromium (^{51}Cr) -release lymphocyte cytotoxicity assays, and by lymphocyte stimulation. Patients gave positive tests in each of these assays. Yet no 1 assay correlated with any of the other 2. Of the 3 assays, only the skin test had some correlation with the clinical status of the patient. As Dr. Halterman described earlier, there was a striking correlation of lymphocyte stimulation with the time after chemotherapy. But so far we had not seen this relationship with the 2 other assays. Do the different assays measure different antigens or do they measure different phases of the immune response?

G. Santos: Two individuals who I suspect were strongly immunized to HL-A antigens (these were 2 sisters that had been multiply pregnant and had multiple transfusions) were strongly positive in the ^{51}Cr-release assay to a sibling's leukemia. This was 60% release, which I have never seen in anyone before, and their migration inhibition factor (MIF) was positive. I wonder if, in a strongly immunized person, we might have a better correlation. For example, in the one family in which we found serum antibody that reacted to the tumor cell (this was the father), the ^{51}Cr- release and the MIF were negative. One of the HI-A-identical siblings was stimulated by the tumor in mixed leukocyte cultures (MLC), but this sibling had no antibody and no ^{51}Cr release. I also mentioned 1 patient in remission who showed a positive response in the MLC to her tumor and a positive ^{51}Cr release but was negative with the MIF and skin test. I don't think there have been enough studies in which these correlations were looked for specifically. The kinds of studies that Dr. Klein was suggesting in the animals need to be examined in greater detail. In animal systems we know that the animal is immune rather than guessing that the patient is immune. In the clinical situation we have the 2 variables (the test and question of immunity); I think we need more animal data.

Herberman: You have raised another important question, Dr. Santos. A lot of us are thinking about some of these assays as a means of monitoring response to therapy, particularly immunotherapy. Although we have restricted most of the discussion to human systems, does anyone have data in animal systems using these types of assays to show that the assays are useful for predicting the response to therapy?

Santos: I have just one, which is the first experiment that I talked about. We gave neuraminidase-treated EL 4 leukemia cells to hybrid mice and had no problem in showing that the animals were immune by challenge. In addition, we demonstrated a positive MIF and a positive ^{51}Cr release against the EL 4 cells.

Herberman: Does anyone else have any data along these lines?

Santos: I am very surprised that there are not more data along these lines. We are asking this question because of clinical interest, and it is within our means to look at known animals. Dr. Klein has at least done a little with this. I think it terribly important, if I may speak as a clinician, to see what these results mean in a known positive system. Here we have 2 unknowns and we don't know what the tests really do or whether the patient is really immune. It is rather foolish to spend so much time, even just looking at the human materials, without examining the animal models.

W. J. Martin: Collaborating with Dr. E. Esber and Dr. J. Wunderlich, I have been investigating the immune response of mice to an injection of irradiated EL 4 leukemia cells. Immunized mouse lymph-node cells are active in a colony inhibition assay; yet these animals show little or no resistance to live tumor challenge. It has been suggested that cells mediating colony inhibition are effector cells in the sense that their in vivo function is to seek and destroy tumor cells. It is then necessary to invoke a suppressive mechanism to explain the lack of tumor resistance. The suggestion is, of course, that blocking antibody negates the activity of these colony-inhibitory cells. An alternative suggestion, however, is that colony inhibition is not always mediated by effector cells but by cells somehow involved in regulating effector cells. Thus the striking feature of EL 4-immunized mice is that, whereas cytotoxic lymphoid cells (CLC) detected in a 4-hour ^{51}Cr-release assay are not present in vivo, these cells will develop during a 5-day in vitro culture.

This finding suggests the existence of an in vivo suppressive mechanism restricting the development of CLC. The question can also be raised whether CLC are true effector cells in vivo. Experiments supporting this notion were presented at the Federation meeting. Thus lymphoid cell suspensions rich in cytotoxic activity could, when transferred to tumor-bearing mice, partially eliminate the tumor cells.

E. Klein: A number of reports indicate that lymphoid cell populations of tumorous animals are less efficient in damaging cells in vitro. We have similar results on primary murine sarcoma virus-induced tumors in the rat. After regression of the tumor, the lymphoid cells show good cytotoxicity. I also would like to remind you about the nonspecific effects of tumorous status demonstrated by Dr. Rapp's group in the guinea-pig hepatoma system; passive transfer of sensitized cells did not inhibit the outgrowth of tumor in animals that carried an unrelated tumor. The same cells were effective in control animals.

Martin: This illustrates the distinction between different types of lymphocytes. One would like to know whether the transferred immune lymphoid cells had cytotoxic activity in vitro. Mouse CLC are often only transiently present in high numbers. The transfer of lymphoid cells at the peak of the cytotoxic response might result in adoptive immunization, whereas the transfer of cells at other times may be ineffective, especially if the environment of the tumor-bearing host was inimical to the proper maturation of precursor cells.

N. Levy: It has puzzled me why brain tumors are not more common. Indeed, several laboratories have shown some diminution in both the efferent and afferent limbs of the immune response in the central nervous system. Meanwhile, we speculate on the importance of the immune system in the host's defense against cancer, while the brain, relatively isolated from immunologic surveillance, remains an infrequent site of neoplasia!

Martin: Perhaps brain cells don't divide as rapidly.

Levy: That is true of only some of the brain's cellular constituents, i.e., neurons and oligodendroglia. Astrocytes and ependymal cells do divide actively and respond with rapid proliferation to various insults to the central nervous system.

Martin: Do glial cells normally divide in vivo?

Levy: Yes.

Martin: What proportion of the brain is glial cells?

Levy: Because of the size of neuronal cells and the large amount of axonal and dendritic mass, the percentage by weight of glial cells would be a misleading figure as to the proportion of central nervous system cells that are glial.

D. Rubin: I'd like to comment on what may be a technical difference in the comparison of some of these tests, for those of you who are concerned whether the test system is complement dependent or not. There is more than one well-established pathway in which complement may play a role in cytotoxicity. Adding carrageenin into your system may not abrogate all of those. It is also known that C1 can be activated without binding to cells and also mediate one of these pathways. As Dr. Levy has said, the effects of specific as well as nonspecific inhibitors should be evaluated before you can say that complement does not play a role in a given in vitro reaction. Perhaps if you looked very closely at the quantitative differences in the results of some of these systems, some may be mediated by complement and some may not. The possible role of complement may be the critical difference and hence be the basis for differences in the interpretation of the results and in the comparison of various tests.

Levy: Another important consideration is that some of the components terminal to C5, or perhaps not involved in the hemolytic sequence at all, may have cleavage products, similar to those of C3 and C5, that are involved in "cell-mediated" cytolysis.

Rubin: If you inactivate C9, there is still some complement activity which affects the cell membrane. You have to go pretty far along to get that, however.

Levy: We must use inhibitors, perhaps artificial substrates, that act terminal to the classic inhibitors like carrageenin, cobra venom factor, etc.

Rubin: The reason that this kind of problem has arisen is because most of you are working in microcytotoxicity plates, where washing the cells in between steps in the overall reaction is not possible. And if you do the serologic tests in tubes where you can wash away the sera before you do the next step, you may eliminate what a lot of people call "nonspecific" inhibition of complement fixation. There is no way around this problem as long as you are going to work in microtiter plates, but you should be aware of it certainly. The AB+ sera could be the source of these kinds of effects. As serum ages, human serum especially, it can acquire biologic activity.

R. Oldham: In reference to your question on correlation of assays, we don't have much evidence on the different kinds of assays, but we do have some evidence that, within the cytotoxicity system, there do seem to be some correlations; i.e., when things have been positive by ^{51}Cr, they also seem to be positive by the iododeoxyuridine (IUDR) system. And if they are positive by IUDR, there also seems to be some correlation with the microcytotoxicity. So I think that within any one type of system there may be some better correlations than between different assay systems.

Herberman: Several people have found that: At some point in time after immunization all the tests are positive, but that's not exactly what I was asking. It is probably more meaningful to look at the kinetics of the immune response by the various assays and correlate the assays with tumor growth or regression and with effects of therapy. It is more difficult to obtain such data, perhaps, but they are not beyond the reach of any laboratory that has set up these tests.

Santos: I was thinking of a graded response. First you do these tests in strongly immunized animals. I will be disturbed if these tests do not all come out positive. Then try to produce different degrees of immunization that can be tested with graded challenge and examine the

relative sensitivities of the tests. There may be one test that always comes through positive, whereas some others drop out. I think that, when we're dealing with the human system, this is important, since we don't know the degree of immunity. Surely a strongly immunized animal has to be tested. But, as Dr. Herberman has said, people have done this, and I think we need to compare a fair number of tests and then at different degrees of immunization (which you test physiologically by challenge). Then you can get into the animal with the tumor during its course. I don't think that type of experiment has been done.

M. Bean: I believe there is somewhat of a difference here between animal and human systems. In the human system, the skin test may be the only test that has been well documented as far as this correlation. Dr. Herberman has said that, in humans, the skin tests seemed to correlate with the clinical disease and the other tests did not. Whereas, in the guinea pig, the skin test did not correlate with the clinical disease.

J. Sinkovics: Could you investigate whether blocking factors could possibly block the positive skin test to a tumor antigen? I mean the delayed-hypersensitivity-type reaction could be blocked in vivo by serum factors.

Herberman: We've talked about it, but we haven't really worked out all of the logistics of getting it done. We plan to test patients whom we know are skin-test positive at a particular time. We will mix the extracts with sera obtained when these patients were skin-test-negative and see if the negative sera will make the skin-test negative. If that works, it will be a direct demonstration that serum blocking can be a factor in the skin tests.

PART VI. CONCLUDING REMARKS

One of the most important aspects of this Conference has been the open and detailed discussion of the problems associated with the assays for cell-mediated immunity to human tumor-associated antigens. A number of reports and claims have been made in the past few years, and it is important to scrutinize these in the light of the present technology. It is becoming increasingly clear, as illustrated by the discussions that we have had, that the performance and interpretation of these assays are quite complex. Several laboratories, using basically the same techniques, have obtained results which differ in several important respects. Hopefully, we have identified some of the technical factors that can influence the results. For the cytotoxicity assays, I was most impressed with the potential differences related to the preparation of lymphocytes and the type of media used.

The first major question we discussed and which is at the center of all our work is: Are some or all of the observed results in an assay due to cell-mediated immunity? It is obviously essential that we be able to differentiate immunologic events from technical artifacts. For example, is an effect due to nonspecific toxicity or feeder effect? Most of us have seen a variety of effects produced by the lymphocytes of normal individuals. Does this represent real immunologic activity against tumor-associated antigens, or is this just noise or problems with setting the baseline in the assays? Finding a completely satisfactory, nonreactive baseline for an assay would be very helpful, but none of those currently used appears to be adequate. For reasons already discussed, a "media control" may be misleading, since it does not measure feeder effects or other nonspecific effects which can occur in the complete assay mixture. Use of a normal individual as baseline also presents a problem, because that excludes the possibility that the normal person may be reactive. If a patient and the normal control are both reactive and to the same extent, this would result in a "negative" test if that normal were considered the baseline.

It is certainly possible that some or all normal individuals have immune reactivity against tumor cells or cell lines derived from tumors. This could be activity against some cross-reactive antigens, e.g., bacterial or histocompatibility antigens. Alternatively, normal individuals might be reacting against tumor-associated antigens. These reactions could play an important role in immunologic surveillance. As proposed by Dr. Thomas and by Dr. Burnet, we may all be developing occult malignancies and rejecting them by our cellular immune response. If so, it should not be surprising to see normal reactivity. There is increasing evidence for normal reactivity to virus-induced antigens in animal systems. Exposure of man to potentially oncogenic agents could also elicit detectable immune reactions to antigens associated with the agents. Dr. Koldovsky suggested earlier in this Conference that there should be some effort to identify normal individuals who are particularly reactive, or unreactive, in the cellular assays. It would be interesting to determine, in a long-term prospective study, whether such people have a higher or lower incidence of cancer.

The specificity of the reactions observed in the various assays is the other issue of great importance. There seem to be several ways to approach the problem: 1) Perform assays with as large a checkerboard of lymphocytes and target cells and controls as is technically possible. By testing a large number of control lymphocytes and control target cells, the specificity of the reactions of patients' lymphocytes against the tumor cells can be better assessed. 2) Inhibition assays have been extremely useful in serologic studies in defining antigenic specificity. Inhibition generally has been more sensitive and precise in resolving antigenic similarities or differences. Similar techniques have not yet been extensively used in cell-mediated assays. Our group has shown that cells bearing the appropriate antigen can inhibit cell-mediated ^{51}chromium release from labeled target cells. Peter Perlmann and Carol O'Toole have shown impressive specificity in their experiments on the urinary bladder cancer system, by inhibiting reactions with extracts of tumor but not with comparable extracts of normal bladder. Dr. Levy referred earlier to an alternative method, of specifically absorbing out reactive lymphocytes on monolayers bearing the appropriate antigens. Extensive utilization of these approaches should provide important information about the specificity of the cellular reactions. This would determine clearly if the reactivity of normal individuals is real and has the same specificity as that of tumor patients. 3) Specificity can also be approached through the blocking or unblocking effects of sera. Will reactive sera affect the cytotoxicity produced by some or all of normal lymphocytes? Can the blocking or unblocking effects in sera be specifically absorbed out by the appropriate tumor cells and not by other types of tumor cells or by normal cells?

Other important problems we have tried to focus on are the interrelationships between the assays and the relation of each assay to the in vivo state of resistance against the tumor. Very little is known about these questions, and I'm sure that is the reason for the very brief discussion we had on this. To reiterate the point that Dr. Santos made earlier, it is important that such questions be asked in the well-defined animal tumor systems. It certainly should be easier to define most or all of the variables in these systems than it is in clinical studies. Animal experiments should be designed which mirror the clinical situations as closely as possible. For instance, it would be most valuable to perform one or more assays for cell-mediated immunity in conjunction with chemotherapy or immunotherapy trials. This would help to determine which assays best reflect the tumor load or prognosis. With human tumors, it would be difficult to say at this moment which assays would be most accurate and practical for monitoring response to therapy. Since clinical immunotherapy is now being contemplated and even actively performed, it will be important to perform some of the assays for cellular immunity as part of the studies. One could then determine which assay correlated best with clinical response or which had the best predictive value.

I am hopeful that this Conference will be only one of a continuing exchange of ideas and information among the groups actively working in this area. Comparison of techniques and also of reagents, such as established cell lines, should help move the field along faster.

Ronald B. Herberman, M.D.
Chairman of the Conference

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